Nitric oxide and abscisic acid regulate osmoprotective and antioxidative mechanisms related to water stress tolerance of grapevines

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Abstract

Background and Aims: Exogenously applied nitric oxide (NO) and abscisic acid (ABA) are known to improve the tolerance of plants to abiotic stress. The effects of NO and ABA applications on physiological and metabolic responses associated with vine protection against water stress were analysed.

Methods and Results: The responses to the NO donor sodium nitroprusside (hereinafter referred to as NO treatment) and ABA were assessed on metabolic profiling, the activity of antioxidant enzymes, and physiological parameters in leaves of water-stressed Malbec vines. Application of NO and ABA partially closed stomata, thus increasing water potential and reducing vine growth. As well, NO and ABA increased guaiacol peroxidase activity and the concentration of specific sugars and anthocyanins in leaves, whereas the accumulation of amino acids was reduced, in turn associated with less protein degradation. Differential responses triggered by NO in stimulating ascorbate peroxidase activity and in incrementing tricarboxylic acid cycle intermediates and terpenes that stabilise membranes, suggest differential mechanisms for NO and ABA in counteracting water stress effects in leaves of grapevines.

Conclusions: Nitric oxide and ABA differentially regulate osmoprotective and antioxidative mechanisms related to water stress tolerance in grapevines.

Significance of the Study: Spray application of NO and ABA to whole plants promoted leaf metabolic responses known to be associated with water stress tolerance mechanisms in grapevines. This approach may provide the basis for improving viticulture in grapegrowing regions affected by water scarcity due to climate change.

Keywords: *abscisic acid, drought, nitric oxide, primary and secondary metabolism,* Vitis vinifera

Introduction

Climate change is likely to increase water scarcity in many grapegrowing regions worldwide (Intergovernmental Panel on Climate Change 2014), which may reduce yield and negatively affect berry composition and wine quality (Chaves et al. 2010, Galat Giorgi et al. 2019). Many studies have explored the molecular (Deluc et al. 2009), metabolic (Castellarin et al. 2007, Grimplet et al. 2009, Savoi et al. 2017) and physiological (Cifre et al. 2005, Chaves et al. 2010, Galat Giorgi et al. 2019) responses of grapevines to water stress. Under mild to moderate water deficits stomatal closure is an early response that restricts water loss and reduces carbon assimilation (Chaves et al. 2010). Under severe or prolonged water deficits other acclimation responses occur, including those related to growth inhibition, reduction of oxidative damage and osmotic adjustment (Chaves et al. 2010, Griesser et al. 2015, Savoi et al. 2017).

The most common compatible solutes, or osmoprotectants, in plants are polyhydroxy compounds (e.g. sucrose, oligosaccharides and polyalcohols) and nitrogen-containing compounds (e.g. proline, other amino acids and polyamines) (Hare et al. 1998). Increases in the concentration of proline (Pro) in grapevine leaves (Doupis et al. 2011), and of branched-chain amino acids besides Pro in grape berries (Deluc et al. 2009, Grimplet et al. 2009, Savoi et al. 2017) have been reported in response to drought stress. Proline has been proposed to act as an osmoprotectant and reactive oxygen species (ROS) scavenger. Furthermore, Pro accumulation could buffer cytosolic pH and balance cell redox status under stress (Verbruggen and Hermans 2008, Hildebrandt et al. 2015). In addition to amino acids, other metabolites, including soluble sugars, polyols and raffinose, have been shown to be involved in response of grapevines to water stress (Grimplet et al. 2009, Conde et al. 2014, Griesser et al. 2015).

Environmental stresses such as drought lead to enhanced generation of ROS in plants due to disruption of cellular homeostasis. Reactive oxygen species are scavenged or detoxified by an antioxidant system comprising enzymatic as well as non-enzymatic antioxidants (Sharma et al. 2012, Kapoor et al. 2019). Phenolic substances and terpenes possess antioxidant properties and may protect against several abiotic stresses (Castellarin et al. 2007, Deluc et al. 2009, Grimplet et al. 2009, Gil et al. 2012). In grape berries, water deficit accelerates ripening and induces changes in gene expression regulating flavonoid biosynthesis, especially the anthocyanin pathway (Castellarin et al.

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2007, Deluc et al. 2009). Furthermore, an increase in the concentration of the monoterpenes, α-pinene, 3-carene, terpinolene and the sesquiterpene nerolidol, all of them with strong antioxidant properties, has been observed for grape leaves in response to mild water deficit (Alonso et al. 2015).

There are numerous studies on the environmental regulation of primary and secondary metabolism in grapevines (Grimplet et al. 2009, Conde et al. 2014, Alonso et al. 2015, Griesser et al. 2015), especially the regulation of polyphenol metabolism in grape berries (Castellarin et al. 2007, Deluc et al. 2009, Savoi et al. 2017). The effects, however, of exogenously applied abscisic acid (ABA) (Alonso et al. 2015, Murcia et al. 2017) and nitric oxide (NO) on metabolism in tissues different from grape berries are not well understood.

Nitric oxide and ABA play an important role as signalling molecules in the physiological responses of plants to several environmental stresses (Zhang et al. 2006, Sami et al. 2018, Del Castello et al. 2019). Plant treatment with NO donors improved biotic and abiotic stress tolerance concomitant with up-regulation of gene expression and activity of antioxidant enzymes (Groß et al. 2013). In maize seedlings, UV-B triggered NO accumulation, which up-regulates gene expression of the phenylpropanoid biosynthetic pathway leading to increased concentration of flavonoids (Tossi et al. 2011, 2012). In grapevine, the close relationship between stomatal conductance and NO accumulation in stomatal guard cells indicates a potential role of this molecule in the drought-signalling pathway (Patakas et al. 2010). Also, NO treatment alleviates accumulation of ROS and membrane lipid peroxidation by the differential activation of antioxidant enzymes in grape skin and pulp (Zhang et al. 2019). Although there are numerous studies on the actions of NO in plants (Freschi 2013, Groß et al. 2013, Sami et al. 2018), most studies reporting the impact of NO on the antioxidant system have been focused on the activity of antioxidant enzymes (Groß et al. 2013). Only a few papers have analysed the NO effects on the profile and concentration of osmoprotective and antioxidant metabolites in plants under stress (Costa-Broseta et al. 2018). To our knowledge, there are no studies that analyse the effect of NO on the metabolome of grapevines either under non-stressful or water stress conditions.

Similar to that reported for NO, exogenously applied ABA can act as a signal that triggers different physiological–biochemical responses associated with plant protection against stress. Pretreatment with ABA of leaf segments caused an increase in the activity of antioxidant enzymes and in the concentration of antioxidant metabolites, thus reducing the oxidative damage in leaves of maize seedlings exposed to water stress (Jiang and Zhang 2002). Few studies have analysed the effect of ABA on grapevine metabolism. Previous studies reported that exogenously applied ABA triggers different metabolic responses associated with protection against abiotic stresses, such as UV-B radiation and mild drought stress (Berli et al. 2010, 2011, Alonso et al. 2015, Murcia et al. 2017). Applications of ABA to grapevine improved tolerance to solar UV-B through an increase in the activity of antioxidant enzymes, accumulation of UV-absorbing compounds and β-sitosterol in leaves (Berli et al. 2010), and accumulation of flavonols and hydroxybenzoic acids in berries (Berli et al. 2011). Applied ABA also elicited defensive mechanisms against mild water deficit by augmenting the concentration of monoterpenes with antioxidant properties in grape leaves (Alonso et al. 2015). It has also been reported that under non-stressful conditions application of ABA evoked synthesis of proline, acidic amino acids and anthocyanins in grape leaves (Murcia et al. 2017).

Interactions between ABA and NO have been suggested to contribute to the regulation of a series of plant adaptative responses to environmental challenges, such as stomatal closure and activation of antioxidant enzymes (García-Mata and Lamattina 2002, Del Castello et al. 2019, Prakash et al. 2019). During the induction of these plant responses, NO would act as a downstream element in the ABA signalling pathway. During the regulation, however, of certain developmental events not linked to plant stress responses, NO appears to act independently of ABA, suggesting a certain level of specificity in the NO–ABA interaction mechanisms, depending on the physiological events under analysis and the type of plant cell considered (Hancock et al. 2011, Freschi 2013).

Vitis vinifera L. cv. Malbec has favourable characteristics for its cultivation in the Andes foothills in Argentina, where it has become the emblematic icon of the country's wine industry. The region has an arid to semi-arid climate, with a temperate or cold-temperate winter season, annual precipitation below 250 mm, altitude ranging from 700 to 1500 masl, and high heliophany throughout the whole year. This study evaluated: (a) the regulatory role of NO (applied as a NO donor) and ABA on primary and secondary metabolism of leaves of Malbec grown under well-watered and water stress conditions; (b) the role of NO and ABA as signalling molecules able to increase the activity of antioxidant enzymes and to induce biochemical changes in vine leaves that ameliorate the deleterious effects of water stress; and (c) whether the effects of NO on leaf antioxidant enzymes activity and metabolism are similar to those observed after ABA application, in regard to the possible role of NO as mediator of ABA signalling.

Materials and methods

Plant material and experimental conditions

Cuttings of *Vitis vinifera* L. cv. Malbec were obtained from 1-year-old cane-pruned vines collected from an experimental vineyard at Estación Experimental Agropecuaria Mendoza-Instituto Nacional de Tecnología Agropecuaria (EEA Mendoza-INTA), Mendoza, Argentina. The cuttings derived from virus-free plants were treated to obtain roots after Murcia et al. (2016). The own-rooted cuttings were then planted in 10 L pots containing grape pomace compost (rich in organic matter and minerals) as substrate. Only one shoot per vine was allowed to grow under field conditions at Instituto de Biología Agrícola de Mendoza (IBAM) (Mendoza, 33°0′S, 68°52′W, 940 masl) during one growing season (2016/17). During establishment, vines were watered to field capacity with tap water (electrical conductivity 944 microS/cm) every 2 days in the morning. The experiment was a completely randomised design with six treatments and ten replicates per treatment (a total of 60 vines). Individual vines were used as experimental units.

The chemical treatments consisted of ABA (ABA treatment), sodium nitroprusside dihydrate (SNP as NO donor, hereinafter referred as NO treatment) and water (Control) solutions every 6 days from 59 days after budburst until the end of the experiment (133 days after budburst). The solutions were sprayed onto the vines until runoff with a hand-held sprayer in the late afternoon for ABA (to minimise photodegradation), and in the morning for NO (to facilitate

NO evolution via a light dependent reaction). The ABA dose was chosen based upon previous studies (Berli et al. 2010, Murcia et al. 2016): 250 mg/L ABA (±-S-*cis*, *trans* ABA, PROTONE SL, Valent BioSciences, Libertyville, IL, USA), and the SNP dose was suggested by Professor Lorenzo Lamattina (pers. commun., November 2013): SNP 60 mg/L (Merck, Darmstadt, Germany), and Control (water), and 0.05% (v/v) Triton X-100 was used as a surfactant. Vines were kept watered to field capacity until the water stress treatment started 45 days after the start of chemical applications. At this time, all the vines were transferred to a greenhouse (Figure S1a, without temperature control) to avoid interference from external precipitation. Inside the greenhouse, midday photosynthetically active radiation (PAR) was 70% of external PAR and temperature averaged 2.5°C higher than outside (Figure S1b). Two irrigation treatments were imposed on two groups of 30 vines each (10 vines, respectively, of Control, NO and ABA): water-stressed vines (D) (stopped watering for 13 days), and well-watered vines (W) irrigated to field capacity every 2 days. At the end of D treatment (117 days after budburst), physiological parameters were measured as indicators of water stress, and adult leaves (9–12th leaves from shoot apex) were collected and kept on dry ice until analysis in the laboratory. There, the leaf petioles were separated and discarded, the length of the main midrib and mass of leaf blades were measured, and then they were stored at −80°C until they were sampled for metabolite analysis. Due to the processing time required for the different biochemical determinations on the same day, from the set of ten plants per treatment eight replicates were randomly used.

Growth parameters

After the D treatment, all the D and W vines were returned to field conditions and re-watered every 2 days to field capacity for 16 days. At the end of the experiment (133 days after budburst), shoot length (SL) and leaf area (LA) were assessed for all vines. Leaf area was estimated after Murcia et al. (2017). Finally, vines were dissected into leaves, shoot and roots, and the dry mass (DM) of these tissues recorded.

Physiological measurements

Stomatal conductance [g_s, mmol $H_2O/(m^2 \cdot s)$] of the 11th leaf from the shoot apex from each vine was measured using a diffusion leaf porometer model SC-1 (Decagon Devices, Pullman, WA, USA). Relative chlorophyll content was measured in nine leaves from the middle section of the shoot toward the apex by using a chlorophyll meter (SPAD-502Plus, Konica Minolta, Osaka, Japan). The results were expressed in arbitrary units, referred here as SPAD units. Stem water potential (Ψ_w) was assessed in one fully expanded mature leaf (13th leaf from the shoot apex) from each vine by using a Scholander Model 2 pressure chamber (BioControl, Buenos Aires, Argentina). Leaves were enclosed in a plastic bag and covered with aluminium foil during 40 min before removing them from the vine. Midday measurements were made between 1200 and 1300.

Protein concentration and antioxidant enzyme activity

Samples of 100 mg leaf fresh mass (FM) were homogenised using a mortar and pestle with 5 mL of extraction solution (100 mmol/L potassium phosphate buffer pH 7.5; 0.1% Triton X-100; 1 mmol/L EDTA; 0.5 mmol/L ascorbic acid) and 125 mg of insoluble polyvinylpolypyrrolidone (PVPP). The mixture was centrifuged at 16 000 *g* for 5 min at 4°C, and the supernatant was used to assess the protein concentration and the enzyme activity. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as the standard. The activity of the enzymes catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POD; EC 1.11.1.7) was measured as described in Berli et al. (2010) with some modifications. The CAT activity was measured by monitoring the consumption of H_2O_2 at 240 nm in 2.5 mL reaction mixture containing 100 mmol/L potassium phosphate buffer (pH 7.5) and 150 µL of enzyme extract. The reaction was started by adding 10 µL of 34.8 mmol/L H_2O_2 and changes in absorbance were monitored for 60 s. The APX activity was measured by the decrease in absorbance of ascorbate at 290 nm in 2.5 mL reaction mixture containing 50 mmol/L potassium phosphate buffer (pH 7.0), 1 mmol/L EDTA, 50 mmol/L ascorbic acid and 1 mmol/L H_2O_2. The reaction was started by adding 75 µL of the plant extract and changes in absorbance were followed for 60 s. The POD activity was measured by monitoring the oxidation of guaiacol at 470 nm in 2.5 mL reaction mixture containing 50 mmol/L potassium phosphate buffer (pH 6.0), 1.2 mmol/L H_2O_2 and 2 mmol/L guaiacol. The reaction was started by adding 100 µL of the enzyme extract and changes in absorbance were monitored for 60 s.

Determination and quantification of free amino acids

The free amino acids (AAs) were separated, identified and quantified according to Murcia et al. (2017). Samples of leaves (100 mg FM) were homogenised with a mortar and pestle in 1 mL of 0.1 mol/L HCL. The suspension was transferred to an Eppendorf tube, with 10 µL of methionine sulfone (1 g/L) as an internal standard. The tubes were shaken for 10 min and then centrifuged for 3 min at 16 000 *g*. The supernatant was purified by solid-phase extraction (SPE), using an Extract Clean SCX (GRACE, Deerfield, IL, USA) 100 mg/1.5 mL cartridge pre-conditioned with 1 mL of 0.1 mol/L HCL and 3 mL of milliQ H_2O. The AAs were eluted with 250 µL of 8 mol/L NH_4OH: methanol (1/1, v/v), and the fraction was collected into a 1.5 mL glass vial. After this step, AAs were derivatised with 10 µL of pyridine and 20 µL of ethyl chloroformate. Then, 90 µL of $CHCl_3$ and 90 µL of 50 mmol/L $NaHCO_3$ were added. Finally, 55 µL from the bottom phase was transferred to an insert for direct injection of 2 µL into a Clarus 500 gas chromatograph equipped with a Clarus 500 single-quadrupole mass spectrometer detector (GC/MS) (PerkinElmer, Shelton, CT, USA).

Determination of soluble sugars and organic acids

Soluble sugars and organic acids were measured according to Murcia et al. (2017) with some modifications. Namely, 1 g of leaf FM was ground to a fine powder and extracted in 5 mL of 0.05 mol/L imidazole: ethanol (50/50 v/v, pH 7) adding β-phenyl-glucopyranoside (50 µL, 2.5 g/100 mL) as internal standard. The mixture was shaken for 18 h at room temperature and then centrifuged 10 min at 7000 *g*. The supernatant was collected and the pellet re-extracted with imidazole solution (5 mL). Aliquots (2 mL) of leaf extracts were dried under gentle N_2, then dissolved in pyridine (150 µL), hexamethyldisilazane (120 µL) and trimethylchlorosilane (30 µL), and heated at 50°C for 1 h. Trimethylsilyl derivates were injected into a Shimadzu GC 2010 Plus GC (Shimadzu, Kyoto, Japan) equipped with a splitter injector, a flame ionisation detector (GC/FID) and a Zebron ZB-1 capillary column, 30 m length, 0.25 mm inner diameter and 0.25 mm film thickness (Phenomenex, Torrance, CA, USA). The

injector and detector temperature was set at 330°C. The column temperature was held at 130°C for 1 min, then increased at 6°C/min to 190°C, at 8°C/min to 250°C, at 25°C/min to 300°C, at 50°C/min to 330°C, and held for 5 min.

Determination and quantification of terpenes

Terpenes were determined according to Pontin et al. (2015) with some modifications. Leaf samples (100 mg FM) were macerated with 1.5 mL of CH_2Cl_2 and 1 mL of methanol: HCO_2H (99.8/0.2, v/v). The macerates were transferred to glass tubes, and extracted overnight in darkness at 4°C. The macerates were centrifuged 5 min at 19 500 *g*, and aliquots (100 µL) of the CH_2Cl_2 phase were put into a GC insert with *n*-hexadecane (100 ng) as the internal standard. Then, 2 µL was injected into the GC/MS (Clarus 500, PerkinElmer). The column was the same as used in the determination of AAs, but in this case the flow rate of carrier gas was 0.7 mL/min. The oven temperature program was set as described in Pontin et al. (2015). The mass spectrometer was operated in electron impact ionisation mode at 70 eV and EI-MS spectra were recorded in the range of 40–500 *m/z* units. The compounds were identified by their fragmentation pattern and comparison with data of the NIST library. Peak areas were referred to the standard *n*-hexadecane for quantification.

Analysis of anthocyanins and low-molecular-mass polyphenols

Anthocyanins and low-molecular-mass polyphenols (LMMPPs) were extracted from leaf tissues after Murcia et al. (2017) with some modifications. One gram of frozen leaf was ground with mortar and pestle, and then macerated with 5 mL of methanol: HCl (99/1, v/v) solution in a glass tube. The extraction was performed by sonication during 30 min at 25°C. Then, the mixture was centrifuged for 10 min at 1300 *g*. The procedure was repeated twice and the supernatants were combined and the extracts made up to 10 mL with extraction solvent.

For determination of anthocyanins, extracts (5 mL aliquots) were individually evaporated to dryness and dissolved in 3 mL of 0.1% HCO_2H in H_2O. Anthocyanins were concentrated by SPE using C_{18} cartridges, and analysed with a Dionex Ultimate 3000 HPLC-DAD system (Dionex Softron, Thermo Fisher Scientific, Germering, Germany) after Murcia et al. (2017). The separation was carried out in a reverse-phase Kinetex C_{18} column (100 mm × 3.0 mm × 2.6 µm) (Phenomenex). The mobile phase consisted of ultrapure H_2O: HCO_2H: acetonitrile (87/10/3, v/v/v; eluent (A) and ultrapure H_2O: HCO_2H: acetonitrile (40/10/50, v/v/v; eluent (B) using the following gradient: 0 min, 10% B; 0–6 min, 25% B; 6–10 min, 31% B; 10–11 min, 40% B; 11–14 min, 50% B; 14–15 min, 100% B; 15–17 min, 10% B; and 17–21 min, 10% B. The mobile phase flow was 1 mL/min, column temperature 25°C, and injection volume 5 µL. The anthocyanin concentration was quantified at 520 nm, and was expressed as malvidin-3-glucoside equivalents by using an external standard calibration curve (1–250 µg/mL, $R^2 = 0.9993$). The identity of the anthocyanins detected with HPLC-DAD was confirmed according to the elution profile and identification of compounds reported in a previous study (Murcia et al. 2017).

For determination of LMMPPs, extract aliquots (5 mL) were evaporated to dryness and dissolved in 5 mL of H_2O. Low-molecular-mass polyphenols were extracted and analysed by HPLC-DAD according to Murcia et al. (2017). Linear ranges between 2 and 40 µg/mL with coefficient of determination (R^2) higher than 0.994 were obtained for all the LMMPPs studied.

Statistical analysis

InfoStat (www.infostat.com.ar) was used for statistical analyses. The effect of ABA and SNP (NO) applications, the water stress treatment and their interactions were assessed by multifactorial ANOVA and the Fishers Least Significant Difference (LSD) test to compare means ($P \leq 0.05$).

Results

Effect of NO and ABA on growth and physiological parameters of vines

Growth and physiological parameters are shown for W and D in Table 1. Drought reduced Ψ_w, and, irrespective of the irrigation treatment, higher values of Ψ_w were observed in NO- and ABA-treated vines in comparison to that of Control vines. Stomatal conductance was reduced by 77% under D, and neither NO nor ABA modified g_s by D, although both NO and ABA promoted stomatal closure in W-treated vines. Drought reduced leaf area and dry mass, as well as shoot length and DM. Regardless of D, NO reduced leaf area, and ABA reduced leaf DM. Root DM was not affected by D or chemical treatments. Chlorophyll concentration of the vine leaves (SPAD index) was increased by D, and again with application of NO and ABA.

Effect of NO and ABA on protein concentration and activity of antioxidant enzymes in vine leaves

Protein concentration was strongly decreased by D; NO partially reversed the D effect, but in W both NO and ABA reduced total protein values (Table 2). The activity of the antioxidant enzyme CAT (catalase) was higher in D vines compared with W vines (33%). The NO treatment reduced the CAT activity under both W and D by 23 and 29%, respectively, while it was reduced by ABA under W by 25%. The NO and ABA treatments increased guaiacol peroxidase (POD) activity by 71 and 89%, respectively under D, and NO increased POD activity under W by 66%. The APX (ascorbate peroxidase) activity was decreased by ABA (relative to NO), while it was not affected by water availability (Table 2).

Effect of NO and ABA on the accumulation of amino acids in vine leaves

The effect of chemical and irrigation treatments on accumulation of free AAs in vine leaves is shown in Table 3. According to the biosynthetic routes, the 13 amino acids identified can be classified as derived from 3-phosphoglycerate (Ser and Gly), pyruvate (Leu, Ala, Val and Ile), oxaloacetate (Asp, Asn and Thr), α-ketoglutarate (Glu, Gln, and Pro) and shikimate (Phe) pathways. The concentration of all AAs was higher in D vines in comparison with W vines, except for Ser, Ile and Gln (Table 3). These results correlate well with the lower total protein concentration measured in D grown leaves compared to the W Control leaves (Table 2), and as D-inhibited growth (Table 1), this implies that proteolysis was predominant under the stress. Nitric oxide and ABA in some cases partially counteracted the proteolytic effect of D. The lowest concentration of Gly was found in ABA-treated vines under D, without difference with that measured in W vines. Under D, vines treated with NO and ABA showed the lower concentration of Phe (52 and 22%,

Table 1. Effect of nitric oxide and abscisic acid on the growth and physiological parameters measured in well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

Treatments	Ψ_w (MPa)	g_s [mmol $H_2O/(m^2 \cdot s)$]	Leaf area (cm^2)	Shoot length (cm)	Root dry mass (g)	Shoot dry mass (g)	Leaves dry mass (g)	SPAD index
Well-watered								
Control	−0.81 ± 0.03b	149.21 ± 9.65a	2656 ± 84.14a	199.28 ± 5.26a	5.01 ± 0.15	11.19 ± 0.59a	9.39 ± 0.24a	32.28 ± 0.33c
Nitric oxide	−0.67 ± 0.03a	78.45 ± 4.77b	2465 ± 60.81a	189.45 ± 5.91a	4.32 ± 0.22	10.49 ± 0.28a	8.77 ± 0.18ab	31.78 ± 0.40c
Absisic acid	−0.59 ± 0.05a	73.31 ± 6.18b	2438 ± 102.0a	190.43 ± 6.53a	4.99 ± 0.27	10.67 ± 0.67a	7.92 ± 0.54b	32.27 ± 0.38c
Water stressed								
Control	−1.28 ± 0.03c	33.72 ± 3.84c	1862 ± 84.66b	144.83 ± 4.38bc	5.03 ± 0.24	7.37 ± 0.21b	6.63 ± 0.33c	34.34 ± 0.36b
Nitric oxide	−0.92 ± 0.08b	42.58 ± 4.42c	1621 ± 64.17c	137.22 ± 3.29c	4.89 ± 0.25	6.88 ± 0.53b	5.77 ± 0.36cd	36.63 ± 0.31a
Abscisic acid	−0.93 ± 0.07b	31.34 ± 2.86c	1771 ± 59.61bc	156.72 ± 4.01b	4.29 ± 0.18	7.78 ± 0.17b	5.54 ± 0.27d	36.25 ± 0.30a
$P_{(I)}$	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.8473	**<0.0001**	**<0.0001**	**<0.0001**
$P_{(C)}$	**0.0001**	**<0.0001**	**0.0174**	0.0913	0.1304	0.3565	**0.0022**	**0.0097**
$P_{(I \times C)}$	0.2054	**<0.0001**	0.5200	0.0964		0.5714	0.6537	**0.0003**

Values are means ± SE, $n = 10$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; P-values ≤ 0.05 are highlighted in bold; different letters indicate a significant difference ($P \leq 0.05$). Ψ_w, stem water potential; g_s, stomatal conductance.

respectively), Ala (75 and 74%), Leu (53 and 27%), Asn (46 and 37%), Asp (46 and 39%) and Glu (43 and 39%) compared with their respective Controls. While the lowest concentration of Thr (44% for NO and 42% for ABA, relative to Control), Val (50% for NO and 33% for ABA, relative to Control), and Ile (46% for NO, relative to Control) were independent of the irrigation treatment (Table 3). Under W, the concentration of Pro in NO-treated vines was about half that of ABA-treated vines, but without significant differences between these chemical treatments compared to the Control. Under D, a 50% decrease in Pro concentration was observed in NO- and ABA-treated compared to the Control leaves. The exception is Ser that was only detected in W vines, and its concentration was lower in the NO treatment (54%) compared to the Control (Table 3).

Effect of NO and ABA on the concentration of carbohydrates and organic acids in vine leaves

Drought dramatically increased the concentration of all the organic acids assessed in the vine leaves, except fumaric and tartaric acids (Table 4). Also, there were statistically significant interactions between chemical and irrigation treatments in the concentration of all organic acids, except quinic acid. Under W, NO and ABA had no effect, except for shikimic acid that was augmented by NO 30-fold with respect to the Control.

Under D, NO increased malic (1.9-fold), fumaric (5.8-fold), succinic (threefold) and tartaric (13.6-fold) acids, and decreased citric (2.2-fold) and ascorbic (6.6-fold) acids, as compared to the Controls. Abscisic acid increased ascorbic acid (1.4-fold) and decreased malic acid (10.3-fold) concentration compared to those measured in D Control vines. The concentration of the remaining organic acids did not change with ABA under D (Table 4).

Drought increased the concentration of all sugars identified in vine leaves (except *myo*-inositol), notably fructose, and there were statistically significant interactions between chemical and irrigation treatments in the concentration of glucose, fructose and sucrose (Table 5). Treatments had no effect whatsoever, except in D conditions where NO decreased the concentration of glucose (sixfold) and sucrose (12-fold), and increased by twofold the concentration of fructose. The ABA treatment stimulated the concentration of fructose (1.5-fold), while reducing sucrose concentration by half. Its effects, however, were of lesser magnitude than those of NO (Table 5).

Effect of NO and ABA on the concentration of di- and triterpenes in vine leaves

Table 6 shows the concentration of di-, triterpenes and sterols measured in vine leaves. The concentration of the sterol precursor squalene was 2.9-fold higher in leaves of D compared to that of the W vines, although phytol and α-tocopherol were also enhanced by 3.4- and 2.5-fold, respectively. The concentration, however, of β-tocopherol and γ-sitosterol was reduced by D. Under W conditions, the concentration of the sterols β-tocopherol, β-sitosterol, γ-sitosterol and ergostenol was significantly lower in NO-treated vines (3.5-, 3.6-, 1.8- and 2.2-fold, respectively, relative to the Control), with no effect of ABA. In contrast, the highest β-sitosterol concentration was measured in NO-treated vines under D (2.9-fold, relative to the Control), without significant difference between the Control and ABA treatments. The concentration of phytol was increased by NO under W (14-fold) and D (2.8-fold), as compared with their respective Controls. Ergostenol was detected

Table 2. Effect of nitric oxide and abscisic acid on the protein concentration and CAT, APX and POD activity measured in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

Treatments	Protein concentration (μg/mg leaf FM)	Activity (nmol/min · mg protein)		
		CAT	APX	POD
Well-watered				
Control	48.29 ± 0.79a	53.54 ± 2.97b	93.37 ± 3.89ab	12.50 ± 1.13c
Nitric oxide	44.87 ± 0.74b	41.28 ± 1.64c	113.47 ± 12.51ab	20.82 ± 3.01b
Absisic acid	41.96 ± 0.69c	40.09 ± 1.86c	81.73 ± 12.31b	15.99 ± 2.45bc
Water stressed				
Control	16.42 ± 1.55e	80.34 ± 4.43a	113.81 ± 14.18ab	17.30 ± 0.88bc
Nitric oxide	20.54 ± 0.60d	57.28 ± 4.42b	130.47 ± 19.63a	29.72 ± 3.90a
Absisic acid	17.78 ± 0.87de	71.71 ± 3.66a	78.38 ± 6.89b	32.79 ± 1.75a
$P_{(I)}$	**<0.0001**	**<0.0001**	0.2871	**<0.0001**
$P_{(C)}$	**0.0126**	**0.0001**	**0.0097**	**0.0002**
$P_{(I \times C)}$	**0.0003**	0.0601	0.6004	0.0753

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; P-values ≤ 0.05 are highlighted in bold; different letters indicate a significant difference ($P \leq 0.05$). APX, ascorbate peroxidase; CAT, catalase; FM, fresh mass; POD, guaiacol peroxidase.

only under W conditions and the NO treatment decreased its concentration 2-fold (Table 6).

The concentration of the triterpene taraxasterol under water stress conditions was 5.2-fold higher in leaves of NO-treated vines (relative to the Control), followed by that measured in ABA-treated vines (2.3-fold). Other treatments had no effect. β-Amyrin, which is a product of the same enzyme oxidosqualene cyclase that catalyses the formation of taraxasterol, was detected only under water stress condition and the NO treatment increased its concentration by 5-fold (Table 6).

Effect of NO and ABA on concentration of anthocyanins and low-molecular-mass polyphenols (LMMPPs) in vine leaves

The concentration of anthocyanins identified in the vine leaves is shown in Table 7. These compounds corresponded to glycosylated forms, delphinidin 3-*O*-glucoside (Del-3-G), petunidin 3-*O*-glucoside (Pet-3-G), cyanidin 3-*O*-glucoside (Cya-3-G), peonidin 3-*O*-glucoside (Peo-3-G), malvidin 3-*O*-glucoside (Mal-3-G) and *p*-coumaroylated-glucosylated forms, peonidin 3-*O*-*p*-coumaroylglucoside (Peo-3-*p* cou), malvidin 3-*O*-*p*-coumaroylglucoside (Mal-3-*p* cou) and cyanidin 3-*O*-*p*-coumaroylglucoside (Cya-3-*p* cou). Water stress increased the concentration of Del-3-G and decreased that of Pet-3-G and Mal-3-G. The concentration of the main identified anthocyanins, Mal-3-G and Peo-3-G, was affected not only by irrigation but also by chemical treatments. While ABA increased the concentration of both anthocyanins, NO application increased only that of Peo-3-G and, in both cases, the increase was independent of vine water status. In contrast, there were significant interactions between chemical and irrigation treatments in the concentration of Peo-3-*p* cou and Mal-3-*p* cou. While the ABA treatment significantly increased the concentration of these anthocyanins under both W and D, NO treatment increased Peo-3-*p* cou and Mal-3-*p* cou concentration only under D. With respect to the anthocyanin Cya-3-G, which possesses a high oxygen radical absorbing capacity (ORAC) (Wang et al. 1997), the highest concentration was measured in the W vines treated with NO. Noticeably, ABA stimulated the glucosyl-coumarylated forms of malvidin, both in W and D vines.

Table 8 shows the LMMPPs characterised from vine leaves in response to the different treatments. These included the nonflavonoids hydroxybenzoic (gallic and syringic) and hydroxycinnamic (caffeic, ferulic and *p*-coumaric) acids, the flavonoids flavanols [(−)-epicatechin, (−)-epigallocatechin gallate], flavanone (naringenin) and flavonols (quercetin-3-glucoside, myricetin-3-glucoside and kaempferol-3-glucoside) and the phenylethanol tyrosol. The concentration of non-flavonoids was generally not affected either by irrigation or by chemical treatments, except ferulic acid (1.5-fold), which was reduced by ABA. The concentration of flavonoids varied, however, in the different treatments. Drought reduced the concentration of (−)-epigallocatechin gallate (8.2-fold) and naringenin (1.9-fold), but increased the concentration of (−)-epicatechin (twofold), and tyrosol (1.8-fold). The effect of ABA application on the concentration of flavonoids was independent of the vine water status, since (−)-epicatechin concentration was increased by ABA under W (4.7-fold) and D (2.1-fold). Contrary to that observed for ABA, the NO effect on the concentration of flavonoids was dependent on vine water status; while NO showed no effects in D, NO treatment increased kaempferol-3-glucoside concentration (2.7-fold), and decreased that of (−)-epigallocatechin gallate (1.9-fold), as compared to their respective Controls under W. The concentration of quercetin-3-glucoside (the second most abundant polyphenol identified in Malbec leaves) was not affected by any of the treatments.

Discussion

Nitic oxide and ABA similarly modify physiological parameters of the vine

It is known that water stress generally reduces plant growth either by decreasing stomatal conductance (g_s) and therefore CO_2 assimilation rate, leaf area (LA), and shoot length (SL), or by disturbing plant osmotic relationships (Cifre et al. 2005, Farooq et al. 2009, Chaves et al. 2010). It was confirmed in our study that water stress (D, drought treatment) led to growth reduction, which was reflected in reduced SL and LA, and lower dry mass of shoot and leaves. This would be the result of a reduced carbon assimilation rate as a consequence of reduced g_s measured in the water-stressed vines. According to the measured g_s values [<50 mmol $H_2O/(m^2 \cdot s)$], the vines experienced severe water stress (Cifre et al. 2005), and under these conditions

Table 3. Effect of nitric oxide and abscisic acid on the concentration of free amino acids in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (ng/mg FM)												
Treatments	**Gly**	**Ala**	**Val**	**Leu**	**Ser**	**Ile**	**Thr**	**Pro**	**Asn**	**Glu**	**Gln**	**Asp**	**Phe**
Well-watered													
Control	6.9 ± 1.1b	139.2 ± 10.4b	438.9 ± 86.5b	387.7 ± 70.7bc	25.1 ± 4.9a	780.6 ± 95.9ab	70.7 ± 8.8bcd	352.6 ± 46.9bc	29.3 ± 0.8c	329.3 ± 38.0c	47.1 ± 11.5	190.1 ± 27.5cd	432.6 ± 81.9c
Nitric oxide	5.1 ± 0.0b	123.4 ± 15.3b	273.3 ± 34.2c	353.4 ± 47.7bc	11.5 ± 1.9b	469.7 ± 53.6c	35.1 ± 7.1d	274.3 ± 31.0c	35.6 ± 5.7c	324.1 ± 48.6c	34.2 ± 4.3	157.3 ± 19.8d	330.4 ± 35.6c
Abscisic acid	5.3 ± 1.0b	175.0 ± 16.4b	353.9 ± 47.6bc	441.9 ± 49.6bc	15.3 ± 2.3ab	625.7 ± 38.3bc	47.6 ± 7.3cd	506.7 ± 54.0b	41.7 ± 6.4bc	440.5 ± 21.2c	66.6 ± 6.6	207.8 ± 16.8cd	466.8 ± 55.3c
Water stressed													
Control	16.6 ± 5.8a	570.9 ± 129.0a	605.6 ± 64.3a	709.4 ± 114.3a	n.d.	1025.2 ± 158.8a	147.1 ± 22.5a	939.4 ± 72.0a	97.0 ± 17.2a	1155.8 ± 116.6a	64.7 ± 9.7	518.8 ± 42.1a	830.7 ± 14.8a
Nitric oxide	9.7 ± 1.7ab	141.3 ± 14.4b	243.9 ± 14.4c	332.5 ± 21.9c	n.d.	495.6 ± 79.3c	90.8 ± 8.8b	478.7 ± 72.1b	52.2 ± 6.5bc	651.5 ± 60.3b	56.7 ± 3.6	280.5 ± 32.2bc	395.2 ± 48.1c
Abscisic acid	3.1 ± 1.0b	147.1 ± 15.3b	349.9 ± 29.7bc	518.1 ± 34.6b	n.d.	933.8 ± 68.1a	72.8 ± 13.7bc	483.5 ± 53.5b	60.8 ± 6.2b	699.3 ± 23.1b	43.9 ± 6.7	316.2 ± 40.3b	643.8 ± 55.1b
$P_{(I)}$	**0.0324**	**0.0019**	0.3069	**0.0240**		**0.0133**	**<0.0001**	**<0.0001**	**0.0001**	**<0.0001**	0.3690	**<0.0001**	**0.0001**
$P_{(C)}$	**0.0079**	**0.0004**	**0.0002**	**0.0116**	**0.0404**	**0.0002**	**0.0012**	**0.0012**	0.1193	**0.0013**	0.3443	**0.0017**	**0.0003**
$P_{(I \times C)}$	**0.0364**	**0.0002**	0.1509	**0.0385**		0.2500	0.1308	**0.0002**	**0.0150**	**0.0002**		**0.0044**	**0.0282**

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; P-values ≤ 0.05 are highlighted in bold; different letters indicate a significant difference ($P \leq 0.05$); n.d., not detected. Ala, alanine; Asn, asparagine; Asp, aspartic acid; FM, fresh mass; Gln, glutamine; Glu, glutamic acid; Gly, glycine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Val, valine.

Table 4. Effect of nitric oxide and abscisic acid on the concentration of organic acids in leaves of well-watered and water-stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (mg/g FM)							
Treatments	**Citric acid**	**Malic acid**	**Fumaric acid**	**Tartaric acid**	**Succinic acid**	**Shikimic acid**	**Quinic acid**	**Ascorbic acid**
Well-watered								
Control	0.068 ± 0.008c	0.017 ± 0.002c	0.027 ± 0.005b	0.043 ± 0.011b	0.042 ± 0.004c	0.086 ± 0.038c	0.020 ± 0.008bc	0.061 ± 0.015c
Nitric oxide	0.132 ± 0.018c	0.018 ± 0.004c	0.034 ± 0.006b	0.025 ± 0.005b	0.033 ± 0.004c	2.744 ± 0.752a	0.020 ± 0.003bc	0.065 ± 0.013c
Abscisic acid	0.145 ± 0.021c	0.015 ± 0.005c	0.028 ± 0.006b	0.017 ± 0.004b	0.027 ± 0.003c	0.045 ± 0.008c	0.012 ± 0.003c	0.043 ± 0.008c
Water stressed								
Control	1.322 ± 0.104a	0.640 ± 0.253b	0.100 ± 0.025b	0.137 ± 0.017b	1.793 ± 0.253b	1.035 ± 0.146b	0.441 ± 0.044a	10.601 ± 0.943b
Nitric oxide	0.589 ± 0.126b	1.226 ± 0.423a	0.583 ± 0.172a	1.871 ± 0.422a	5.344 ± 2.558a	0.454 ± 0.107bc	0.231 ± 0.038b	1.607 ± 0.804c
Abscisic acid	1.186 ± 0.239a	0.062 ± 0.020c	0.177 ± 0.056b	0.178 ± 0.037b	2.291 ± 0.373b	0.986 ± 0.274b	0.582 ± 0.134a	15.089 ± 2.166a
$P_{(I)}$	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	0.5986	**<0.0001**	**<0.0001**
$P_{(C)}$	**0.0089**	**0.0124**	**0.0004**	**<0.0001**	**0.0042**	**0.0025**	0.0747	**<0.0001**
$P_{(I \times C)}$	**0.0033**	**0.0127**	**0.0005**	**<0.0001**	**0.0041**	**0.0001**	0.0595	**<0.0001**

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; P-values ≤ 0.05 are highlighted in bold, different letters indicate a significant difference ($P \leq 0.05$). FM, fresh mass.

Table 5. Effect of nitric oxide and abscisic acid on the concentration of free sugars in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (mg/g FM)						
Treatments	**Ribose**	**Mannose**	**Galactose**	**Glucose**	**Fructose**	**Sucrose**	***myo*-Inositol**
Well-watered							
Control	0.011 ± 0.001c	0.10 ± 0.04b	0.13 ± 0.01b	2.99 ± 0.43b	0.40 ± 0.03d	2.28 ± 0.60bc	4.27 ± 0.33bc
Nitric oxide	0.011 ± 0.002c	0.41 ± 0.12b	0.12 ± 0.01b	2.55 ± 0.29b	0.17 ± 0.04d	1.71 ± 0.32bcd	3.60 ± 0.45c
Abscisic acid	0.012 ± 0.002c	0.06 ± 0.02b	0.13 ± 0.01b	3.21 ± 0.42b	0.06 ± 0.01d	1.11 ± 0.10cd	3.85 ± 0.44bc
Water stressed							
Control	0.22 ± 0.03ab	5.99 ± 0.86a	0.42 ± 0.14a	18.86 ± 2.08a	11.30 ± 1.37c	5.38 ± 0.46a	4.96 ± 0.53abc
Nitric oxide	0.14 ± 0.04b	6.55 ± 1.09a	0.30 ± 0.10ab	3.13 ± 1.42b	24.26 ± 5.45a	0.44 ± 0.14d	6.40 ± 2.49ab
Abscisic acid	0.25 ± 0.05a	6.04 ± 1.12a	0.27 ± 0.03ab	20.70 ± 3.70a	17.53 ± 1.15b	2.42 ± 0.90b	7.38 ± 1.15a
$P_{(I)}$	**<0.0001**	**<0.0001**	**0.0008**	**<0.0001**	**<0.0001**	**0.0103**	**0.0032**
$P_{(C)}$	0.2887	0.8154	0.5109	**<0.0001**	**0.0246**	**<0.0001**	0.5188
$P_{(I \times C)}$	0.3009	0.9874	0.5315	**<0.0001**	**0.0198**	**0.0003**	0.2568

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; P-values ≤ 0.05 are highlighted in bold, different letters indicate a significant difference ($P \leq 0.05$). FM, fresh mass.

NO and ABA application had no effect on g_s. Both NO and ABA treatments, however, reduced g_s under no water stress. Under non-stressful conditions, García-Mata and Lamattina (2002) showed that both SNP (as NO donor) and ABA applications induced stomatal closure, and NO accumulation in *Vicia fava* guard cells was necessary for the ABA-induced stomatal closure. In leaves of drought stressed-grapevines, increase in the concentration of endogenous ABA and NO has been correlated with stomatal closure (Patakas et al. 2010). In our study, however, application of NO and ABA induced stomatal closure only under well-watered conditions and not under water stress. It is possible that the water stress treatment imposed was too severe [apart from the water deficit applied, environmental drought might be magnified by high temperature combined with low RH (see Figure S1b)], and that the endogenous concentration of these regulators was high enough to induce stomata closure, and higher concentration of NO and consequently ABA (exogenously applied) would not have additionally affected this response. In this sense, many studies have shown that complex signal transduction pathways in guard cells are involved in the regulation of stomatal aperture (Sami et al. 2018), and even dual and compensatory mechanisms exerted by NO in the promotion and attenuation of the ABA-induced/phospholipid-mediated signals triggering the stomatal closure have been suggested (Laxalt et al. 2016).

Water stress reduced Ψ_w, and, regardless of the irrigation treatment, higher values of Ψ_w were observed in NO- and ABA-treated vines, which was closely related to NO- and ABA-induced stomatal closure. Stomatal closure reduces transpirational water losses, but also limits CO_2 uptake by leaves and, therefore, photosynthesis. Although the net CO_2 fixation was not measured in our study, the smaller LA measured in NO-treated vines, and the reduced dry mass of leaves from ABA-treated vines, suggest a reduced CO_2 assimilation rate in part because g_s was reduced.

A lower concentration of chlorophyll during drought stress has been reported in wheat plants (Yadav et al. 2019). In our study, the chlorophyll concentration was increased in vine leaves under water stress (possibly as result of a concentration effect by reduced LA), and even more with NO and ABA applications. In contrast, the chlorophyll concentration remained unchanged in response to NO and ABA applications under well-watered conditions. These results agree with those reported by Sahay et al. (2019), who showed that application of SNP and ABA affected the chlorophyll concentration in *Brassica juncea* leaves mainly under stress conditions. Similarly, exogenous ABA increased chlorophyll concentration in drought-stressed leaves, but not in well-watered leaves of tea plants (Zhou et al. 2014). Also, it has been hypothesised that NO could mediate chlorophyll protection through their capability to scavenge ROS (Del Castello et al. 2019).

In our study, the application of NO and ABA reduced stomatal conductance and improved the water status of vines, but reduced the vine growth (expressed as a smaller leaf area and dry mass of leaves).

Nitric oxide and ABA differentially regulate the activity of antioxidant enzymes in vine leaves

One main factor that impairs plant growth and productivity during drought stress is the enhancement of ROS production in organelles, namely, chloroplasts, mitochondria and peroxisomes (Farooq et al. 2009). To cope with stress, plants have a complex antioxidant defence system, comprising enzymatic and non-enzymatic antioxidants (Kapoor et al. 2019). In our experiments, the enzymatic response of droughted leaves to oxidative stress is represented by enhancement of the H_2O_2-scavenging enzyme CAT.

It has been reported that NO stimulates gene expression and activity of antioxidant enzymes such as superoxide dismutase (SOD), CAT and APX under non-stressful conditions, and this effect is enhanced under stress (Groß et al. 2013). In tablegrapes, NO treatment alleviated ROS accumulation and membrane lipid peroxidation by activation of SOD, APX, CAT, POD and glutathione reductase (GR) activity in skin and pulp, delaying postharvest senescence of grapes (Zhang et al. 2019). Our results showed that NO increased POD but reduced CAT activity. Similar effects were reported in nitric oxide synthase-expressing tobacco plants, which exhibited decreased CAT and increased POD activity, without changes in APX activity (Chun et al. 2012).

It has been reported that exogenous ABA induced the activity of SOD, CAT and APX in maize plants (Jiang and Zhang 2001). In contrast, our results showed that ABA increased POD, and decreased CAT and APX activity. The role of NO as mediator of ABA induction of several antioxidant enzymes such as GR (glutathione reductase), SOD, APX and CAT has been reported (Freschi 2013). The

Table 6. Effect of nitric oxide and abscisic acid on the concentration of diterpenes, triterpenes and sterols in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (ng/mg FM)										
Treatments	**Phytol**	**α-Tocopherol**	**β-Tocopherol**	**Squalene**	**β-Amyrin**	**Taraxasterol**	**β-Sitosterol**	**γ-Sitosterol**	**Stigmasterol**	**Fucosterol**	**Ergosterol**
Well-watered											
Control	51.7 ± 6.1d	318.7 ± 62.6c	24.5 ± 4.5a	102.5 ± 15.9c	n.d	27.8 ± 6.3c	21.9 ± 2.8b	1016.3 ± 145.5a	28.2 ± 3.7	37.2 ± 5.2	13.4 ± 1.5a
Nitric oxide	738 ± 61.4a	403.7 ± 66.3c	7.3 ± 1.4b	68.1 ± 13.2c	n.d	16.8 ± 1.4c	6.1 ± 1.4c	560.6 ± 53.3b	21.9 ± 3.6	20.9 ± 3.5	6.2 ± 1.2b
Abscisic acid	102.1 ± 8.3cd	433.7 ± 41.9c	21.5 ± 3.1a	110.6 ± 9.7c	n.d	34.0 ± 6.2c	14.7 ± 1.3bc	879.5 ± 67.4a	33.5 ± 2.9	42.8 ± 6.3	12.1 ± 1.0a
Water stressed											
Control	173.4 ± 19.1c	791.2 ± 68.9ab	9.1 ± 1.0b	295.1 ± 26.4b	4.9 ± 0.9b	76.2 ± 10.3c	12.9 ± 2.1bc	265.9 ± 14.0c	11.9 ± 1.7	10.9 ± 1.4	n.d.
Nitric oxide	483.6 ± 74.3b	1037.3 ± 204.7a	15.1 ± 4.5ab	383.3 ± 50.7a	24.8 ± 6.2a	397.0 ± 78.3a	37.9 ± 12.1a	359.2 ± 75.1bc	33.1 ± 8.0	48.4 ± 10.4	n.d.
Abscisic acid	134.9 ± 21.4cd	537 ± 66.6bc	10.9 ± 2.5b	335.5 ± 38.6ab	6.8 ± 1.1b	177.7 ± 35.1b	20.5 ± 2.8b	183.6 ± 37.0c	25.6 ± 4.6	17.2 ± 3.2	n.d.
$P_{(I)}$	0.2950	**<0.0001**	**0.0258**	**<0.0001**		**<0.0001**	**0.0147**	**<0.0001**	0.2259	0.0840	
$P_{(C)}$	**<0.0001**	0.0536	0.1854	0.6224	**0.0007**	**<0.0001**	0.5600	0.0889	0.0793	0.2143	**0.0128**
$P_{(I \times C)}$	**0.0001**	**0.0263**	**0.0026**	0.1200		**<0.0001**	**0.0005**	**0.0030**			

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; *P*-values ≤ 0.05 are highlighted in bold; different letters indicate a significant difference ($P \leq 0.05$); n.d., not detected. FM, fresh mass.

Table 7. Effect of nitric oxide and abscisic acid on the concentration of anthocyanins in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (μg/g FM)							
Treatments	**Del-3-G**	**Pet-3-G**	**Cya-3-G**	**Peo-3-G**	**Mal-3-G**	**Peo-3-*p* cou**	**Mal-3-*p* cou**	**Cya-3-*p* cou**
Well-watered								
Control	0.07 ± 0.01b	0.23 ± 0.04a	0.11 ± 0.04b	1.38 ± 0.09cd	2.75 ± 0.39bc	0.09 ± 0.01d	0.25 ± 0.06b	n.d
Nitric oxide	0.06 ± 0.01b	0.28 ± 0.06a	0.45 ± 0.20a	3.21 ± 0.54a	3.18 ± 0.26b	0.40 ± 0.02cd	0.31 ± 0.06b	n.d
Abscisic acid	0.09 ± 0.01ab	0.27 ± 0.03a	0.14 ± 0.01b	2.81 ± 0.16ab	4.46 ± 0.27a	0.50 ± 0.08c	1.07 ± 0.18a	0.22 ± 0.03
Water stressed								
Control	0.10 ± 0.01a	0.07 ± 0.01b	0.11 ± 0.01b	0.85 ± 0.05d	1.83 ± 0.12d	0.30 ± 0.04cd	0.59 ± 0.06b	n.d
Nitric oxide	0.08 ± 0.01ab	0.09 ± 0.01b	0.16 ± 0.02b	2.04 ± 0.14bc	2.13 ± 0.14cd	1.16 ± 0.17a	1.16 ± 0.15a	n.d
Abscisic acid	0.09 ± 0.01ab	0.09 ± 0.01b	0.20 ± 0.03b	2.24 ± 0.10bc	3.00 ± 0.22b	0.84 ± 0.11b	1.19 ± 0.23a	0.17 ± 0.03
$P_{(I)}$	**0.0328**	**<0.0001**	0.1404	**0.0026**	**<0.0001**	**0.0001**	**0.0004**	0.2828
$P_{(C)}$	0.3565	0.4212	**0.0157**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**	
$P_{(I \times C)}$	0.3668	0.9023	**0.0300**	0.4319	0.5493	**0.0430**	**0.0292**	

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; *P*-values ≤ 0.05 are highlighted in bold, different letters indicate a significant difference ($P \leq 0.05$); n.d., not detected. Cya-3-*p* cou, cyanidin *O*-*p*-coumaroylglucoside; Cya-3-G, cyanidin 3-*O*-glucoside; Del-3-G, delphinidin 3-*O*-glucoside; FM, fresh mass; Mal-3-*p* cou, malvidin 3-*O*-*p*-coumaroylglucoside; Mal-3-G, malvidin 3-*O*-glucoside; Peo-3-G, peonidin 3-*O*-glucoside; Peo-3-*p* cou, peonidin 3-*O*-*p*-coumaroylglucoside; Peo-3-G, petunidin 3-*O*-glucoside.

Table 8. Effect of nitric oxide and abscisic acid on the concentration of low-molecular-mass polyphenols in leaves of well-watered and water stressed *Vitis vinifera* cv. Malbec vines.

	Concentration (µg/g FM)										
Treatments	**Gallic acid**	**Tyrosol**	**Syringic acid**	**(−)-Epicatechin**	**(−)-Epigallocatechin gallate**	**Naringenin**	**Caffeic acid**	***p*-Coumaric acid**	**Ferulic acid**	**Myricetin**	**Quercetin**	**Kaempferol**
Well-watered												
Control	2.26 ± 0.37	2.62 ± 0.30c	1.84 ± 0.12	14.29 ± 2.67d	5.25 ± 0.46a	4.10 ± 0.87ab	6.58 ± 0.66a	1.50 ± 0.12	4.42 ± 0.66ab	5.91 ± 0.87a	34.64 ± 3.49	1.71 ± 0.71b
Nitric oxide	2.15 ± 0.23	4.12 ± 0.57bc	2.03 ± 0.22	39.50 ± 6.32cd	2.71 ± 0.81bc	2.27 ± 0.18c	5.84 ± 1.37a	2.26 ± 0.08	5.25 ± 0.58a	4.42 ± 1.10ab	36.25 ± 2.48	4.65 ± 0.90a
Abscisic acid	2.22 ± 0.25	3.67 ± 0.46c	1.96 ± 0.15	66.66 ± 8.52bc	3.80 ± 0.58ab	5.60 ± 0.81a	5.56 ± 0.78ab	1.69 ± 0.17	2.83 ± 0.30c	5.71 ± 0.98ab	35.31 ± 1.06	1.14 ± 0.17b
Water stressed												
Control	2.43 ± 0.33	6.46 ± 0.31ab	1.93 ± 0.15	55.70 ± 5.78bc	0.64 ± 0.13d	1.81 ± 0.20c	5.07 ± 1.14ab	1.92 ± 0.17	4.51 ± 0.34ab	4.17 ± 0.37ab	33.02 ± 4.35	1.63 ± 0.25b
Nitric oxide	1.93 ± 0.34	4.51 ± 0.83bc	2.09 ± 0.19	74.65 ± 10.75b	0.62 ± 0.04d	1.71 ± 0.35c	4.61 ± 0.57ab	1.72 ± 0.15	3.57 ± 0.27bc	3.79 ± 0.55b	32.06 ± 3.20	1.17 ± 0.27b
Abscisic acid	3.55 ± 0.66	7.62 ± 1.50a	2.45 ± 0.29	115.04 ± 20.58a	1.92 ± 0.62cd	2.58 ± 0.43bc	2.80 ± 0.62b	1.70 ± 0.25	3.06 ± 0.45c	4.28 ± 0.38ab	32.61 ± 4.25	0.70 ± 0.34b
$P_{(I)}$	0.2069	**0.0007**	0.1934	**0.0001**	**<0.0001**	**0.0002**	**0.0246**	0.8179	0.2419	**0.0413**	0.3401	**0.0115**
$P_{(C)}$	0.1317	0.2692	0.2676	**0.0001**	**0.0481**	**0.0033**	0.2336	0.2348	**0.0049**	0.3738	0.9956	**0.0088**
$P_{(I \times C)}$		0.0802		0.8284	**0.0344**	0.0917	0.6949		0.0811	0.7347		**0.0094**

Values are means ± SE, $n = 8$; $P_{(I)}$, effect of irrigation treatments; $P_{(C)}$, effect of chemical treatments; $P_{(I \times C)}$, interaction effect; *P*-values ≤0.05 are highlighted in bold; different letters indicate a significant differences ($P \leq 0.05$). FM, fresh mass.

differential regulation of APX by NO suggests, however, that the ABA effect in vines is not necessarily dependent on NO as a signalling intermediary.

Nitric oxide and ABA differentially reduce water stress-induced accumulation of free amino acids in vine leaves

A common response of plants to drought is the accumulation of specific AAs that serve as osmolytes, alternative respiratory substrates, ROS scavengers, as well as potential regulatory and signalling molecules (Hildebrandt et al. 2015). Our results demonstrated an accumulation of a large amount of AAs in water-stressed vine leaves, which was consistent with previous reports in other grapevines cultivars showing increased amounts of Leu, Val, Ile, Thr and Pro in berries of drought-stressed grapevines (Grimplet et al. 2009, Doupis et al. 2011, Savoi et al. 2017). Increased concentration of virtually all AAs, rather than increased concentration of specific AAs in vine leaves, would indicate enhanced protein degradation and/or decreased protein biosynthesis as a result of water stress. During abiotic stress or leaf senescence, metabolism shifts from anabolism to catabolism. In this sense, the lowest concentration of total proteins was measured in the water-stressed leaves, which could be associated with leaf senescence as it has been reported by Less and Galili (2008).

Modolo et al. (2006) reported that Arabidopsis NO-deficient *nia1nia2* mutant plants accumulated a low concentration of endogenous NO and free AAs, particularly L-arginine. It is important to keep in mind, however, that these mutants are impaired in nitrate reductases (NR1/NIA1 and NR2/NIA2), essential enzymes for the assimilation of nitrogen in plants. Also, Gupta et al. (2012) reported that NO inhibits aconitase under hypoxia, which results in accumulation of citrate, leading to a shift of the metabolism of Arabidopsis seedlings towards AA biosynthesis. In our experiment, NO treatment did not affect the concentration of free AAs under well-watered conditions (except Ser), although NO reduced drought-induced accumulation of almost all free AAs (Ala, Gly, Lue, Asn, Asp, Phe, Pro) to the concentration measured in well-watered Control leaves. The reduced concentration of Val, Ile and Thr measured in NO-treated leaves was independent of irrigation treatments, consistent with the negative regulatory role of NO on the concentration of some AAs such as Thr and Tyr (Boldizsár et al. 2013). Recent observations showed that the concentration of Asn was negatively associated with drought tolerance in wheat (Yadav et al. 2019), consistent with the fact that Asn accumulates in senescent leaves. Also, NO has been reported as an anti-senescence agent (Sami et al. 2018). The results obtained in the present study suggest lower accumulation of osmolytes due to a better water status of NO-treated vines under water stress. Furthermore, a higher total protein concentration of these vines (in relation to water stressed Controls), and Asn concentration similar to that of well-watered Controls, suggest less protein degradation associated with delayed foliar senescence in NO-treated vines. With respect to ABA, the over-expression of *ABF3* (ABA responsive element-binding factor 3) in soybean plants, which improves tolerance to drought, led to accumulation of free AAs in response to moderate drought (Nam et al. 2019). The results provided here, however, suggest that, as in NO-treated vines, the better water status of ABA-treated vines might explain, in part, the lower concentration of almost all free AAs (Ala, Gly, Lue, Asn, Glu, Asp, Phe,

Pro) measured in ABA-treated vines, compared to Control vines under water stress.

Proline is known to accumulate under stress and considered to act as a compatible osmolyte and radical scavenger (Hildebrandt et al. 2015). In our work, increased Pro was found in leaves of water-stressed Control vines. The NO and ABA co-treatments reduced (by 50%) water stress-induced accumulation of Pro to that concentration measured in well-watered Control leaves. Although there were differences in the Pro concentration between NO- and ABA-treated vines under well-watered conditions, they did not differ from the Control. In agreement with our results, exogenous NO application under non-stressful conditions was unable to stimulate expression of genes encoding Pro-metabolising enzymes or proline production in *Medicago truncatula*, and also induced a significant decrease of Pro concentration in response to abiotic stress in other plants species (Planchet et al. 2014). In summary, the increase in Pro as an osmoprotectant and ROS scavenger does not appear to be a biochemical mechanism by which both NO and ABA could protect macromolecular sub-cellular structures from oxidative damage caused by severe water stress in vine leaves. The activation of the antioxidant enzymes mainly by NO application might be a more efficient mechanism than Pro to scavenge high ROS concentration produced under drought as was proposed by Doupis et al. (2011).

Nitric oxide and ABA increase the concentration of fructose but only NO affects the concentration of organic acids in water-stressed leaves

In addition to the contribution of AAs to osmotic adjustment, the concentration of organic acids and sugars in the vine leaves increased under water stress. Accumulation of organic acids of the tricarboxylic acid cycle (TCA), such as citric, succinic and malic acids, as well as ascorbic and quinic acids, was observed in the water-stressed leaves. This agrees with previous studies showing that some plant species can resist drought by releasing organic acids, such as citrate, oxalate, succinate, tartrate and malate (Griesser et al. 2015, Ullah et al. 2017). Nitric oxide treatment did not affect intermediates of the TCA cycle in leaves of well-watered grapevines, but improved the accumulation of succinic, fumaric and malic acids under water stress. In contrast, the co-treatment with ABA affected only malic acid concentration, decreasing it in the water-stressed leaves. These results are consistent with previous studies (Gupta et al. 2012, Costa-Broseta et al. 2018, Zhao et al. 2019) that showed in plant species other than *V. vinifera*, the concentration of succinate, fumarate and malate decreased in the absence of NO under hypoxia and drought, and also in a NO-deficient mutant of *Arabidopsis*. As well, an elevated concentration of succinate correlated with the efficient use of the TCA cycle to produce more energy under water-limited conditions (Ullah et al. 2017). Furthermore, it is known that malate regulates the redox balance in different plant cell compartments and acts as an osmolyte and anion, compensating the positive charge of potassium, particularly important in stomatal responses (Igamberdiev and Eprintsev 2016).

The accumulation of organic acids does not appear to be a common response triggered by NO and ABA to reduce the deleterious effects of drought. Under water stress, while the lowest concentration of ascorbic acid was measured in NO-treated, the highest was measured in ABA-treated vines. This opposite effect of NO and ABA on the ascorbic acid concentration may be due to the NO-increased APX activity, an enzyme that uses ascorbate to reduce H_2O_2 to H_2O, and the high concentration of the strong antioxidant tartaric acid produced from ascorbic acid catabolism.

The increase in soluble sugars, such as glucose, galactose, mannose, fructose, sucrose, and *myo*-inositol, among others, has been reported in grapevines under drought (Grimplet et al. 2009, Griesser et al. 2015). But there is no information about sugar metabolism in water-stressed vines sprayed with NO and ABA. Under non-stressful conditions, total soluble carbohydrates (glucose, fructose and sucrose) are estimated to represent approximately 70% of the osmotically active solutes in young vine leaves (Patakas 2000). These sugars play an important role as osmoprotectants, antioxidants and in maintaining leaf function and photosynthesis during drought stress (Deluc et al. 2009). According to this, the concentration of almost all sugars identified in vine leaves was increased under water stress. When water-stressed vines, however, were co-treated with NO or ABA, only fructose concentration increased. Thus, the increase in the reducing monosaccharide fructose appears to be a defensive mechanism induced by both NO and ABA against water losses. Similar results were reported for transgenic soybeans (Nam et al. 2019), that over-expression of *ABF3*, which confers improved tolerance to drought, did not have a significant impact on sugar metabolism, except for an increase in fructose under mild-water deficit.

Nitric oxide differentially regulates the concentration of diterpenes, triterpenes and sterols depending on the water status of the vines

It is generally accepted that the maintenance of integrity and stability of membranes under water stress is a major component of drought tolerance in plants (Farooq et al. 2009). In general, the cytosolic mevalonic acid (MVA) pathway is responsible for the synthesis of sesquiterpenes, triterpenes and sterols, whereas the plastidial methylerythritol phosphate (MEP) pathway furnishes the diterpenes and tocopherols (Croteau et al. 2000). In plants, both sterols and triterpenes are synthesised from the common precursor squalene. Alonso et al. (2015) reported squalene accumulation in grapevine leaves subjected to mild water stress. In our work, squalene concentration was affected only by irrigation treatments, being more abundant in the leaves of water-stressed vines. The increase in squalene, however, was not associated with large increases in the membrane concentration of sterols and triterpenes. Thus, water stress stimulated only the biosynthesis of α-tocopherol, while it reduced that of β-tocopherol and γ-sitosterol. Thus, it has been suggested that biosynthesis of triterpenes occurs when sterol formation has been sacrificed (Kamisako et al. 1984). Tocopherols are diterpenes with antioxidant properties that protect lipids from peroxidation and physically stabilise membrane structures by modulating membrane fluidity (Wang and Quinn 2000, Mène-Saffrané and DellaPenna 2010). Thus, the accumulation of α-tocopherol in vine leaves could be a mechanism to protect the membranes against peroxidation and water stress induced-ROS.

The effect of NO on the biosynthesis of sterols, di- and triterpenes in vine leaves was dependent on the irrigation treatments. Under well-watered condition, NO treatment decreased the concentration of the sterols (β-sitosterol, γ-sitosterol and ergosterol) and the diterpene β-tocopherol.

While under water stress, NO significantly increased the concentration of β-sitosterol, phytol and the triterpenes taraxasterol and β-amyrin. Sterols, in addition to regulating fluidity and permeability of the membranes, have shown capacity to intercept free radicals. Recently, Li et al. (2019) reported that application of β-sitosterol could improve drought tolerance of white clover enhancing the accumulation of metabolites associated with growth maintenance, osmotic adjustment and antioxidant capacity.

Another diterpene related to antioxidant defence is phytol, which is known to be required for tocopherol synthesis (Almeida et al. 2016), and also having lipophilic antioxidant properties. A higher concentration of phytol was measured in NO-treated vines under both well-watered and water stress conditions, as compared with their respective Controls. The increased concentration of phytol suggests that NO might help protect the photosynthetic apparatus under water stress conditions, avoiding propagation of lipid peroxidation in thylakoid membranes. Unlike the NO-induced changes in terpene biosynthesis, the lack of response to ABA applications suggests that ABA effects in vines are not necessarily dependent on NO as signalling intermediary.

Nitric oxide and ABA stimulate biosynthesis of anthocyanins instead of low-molecular-mass polyphenols in vine leaves

Among the non-enzymatic antioxidants that protect the plant cell against ROS are phenolic substances, which can be divided into two categories, flavonoids and non-flavonoids. Anthocyanins are the most relevant flavonoids and play important physiological functions in vegetative organs, such as roots and leaves, where they accumulate in response to several biotic and abiotic stress conditions, acting as general antioxidants against ROS and signalling molecules (Wang et al. 1997, Braidot et al. 2008a). It has been reported that water deficit increases synthesis of anthocyanins and expression of flavonoid transporters in grape berries (Castellarin et al. 2007, Braidot et al. 2008b). We did not find relevant changes, however, in the accumulation of anthocyanins in water-stressed leaves, even when the concentration of their biosynthetic precursor Phe was significantly high in these organs.

The water-stressed leaves showed only an increased concentration of Del-3-G and a decreased concentration of Pet-3-G, Peo-3-G and Mal-3-G, probably as a consequence of lower *O*-methyltransferase (OMT) activity. In this sense, a decrease in the proportion of methoxylated anthocyanins from both the F3′H and F3′5′H branches of the anthocyanin pathway was observed in grape berries in response to the partial rootzone drying (PRD) treatment, suggesting reduced 3′*O*-methyltransferase and 5′*O*-methyltransferase activity (Bindon et al. 2008).

The most abundant anthocyanins increased when vines were treated with NO or ABA. The NO and ABA-induced increase of the non-acylated anthocyanins (Peo-3-G and Mal-3-G), was independent of the water status of the vines. While ABA treatment increased both anthocyanins, NO increased only Peo-3-G. In a previous study, we reported that ABA application increased anthocyanins in grapevine leaves under non-stressful conditions (Murcia et al. 2017). Furthermore, ABA application increased non-acylated anthocyanins in Malbec berries (Berli et al. 2011). In the case of *p*-coumaroylated anthocyanins (Peo-3-*p* cou and Mal-3-*p* cou), ABA treatment increased both under well-watered and water stress conditions, while NO treatment increased their concentration only under water stress. Tossi et al. (2011, 2012) demonstrated in maize that NO production is required for UV-B light-induced accumulation of total anthocyanins and flavonoids, and that NO enhances plant UV-B protection up-regulating gene expression of phenylpropanoid biosynthetic pathway. Therefore, it can be suggested that like ABA, NO could counteract the deleterious effects produced by drought in vines by inducing the production of anthocyanins, compounds well known for their ability to scavenge ROS (Wang et al. 1997). The most oxidised (trihydroxylated and methoxylated) anthocyanins have less antioxidant power, whereas the ORAC of Cya-3-G is the highest (Wang et al. 1997). In this regard, the highest concentration of Cya-3-G was measured in well-watered vines treated with NO, while Cya-3-*p* cou was detected only in ABA-treated vines.

Nitric oxide and ABA treatments differentially regulated the accumulation of specific non-anthocyanin phenols. While ABA application increased the concentration of (−)-epicatechin and naringenin independently of the vine water status, the NO-induced increase in the concentration of kaempferol-3-glucoside was dependent on vine water status.

In summary, although application of NO and ABA modified the amount of anthocyanins and non-anthocyanins in vine leaves, the NO treatment did not produce the same effect on the accumulation of these antioxidant compounds as the ABA treatment, which could indicate that the ABA signal is not necessarily dependent on NO but other intermediaries are likely involved.

Conclusions

To our knowledge, this is the first report investigating the comparative effects of exogenously applied NO and ABA on secondary metabolite profiles and antioxidant enzyme activities in grapevines under non-stressful conditions and under water stress. Under water stress, vines showed increased CAT activity and accumulated metabolites associated with osmotic adjustment and antioxidant protection, such as free AAs, organic acids, sugars, flavanols as (−)-epicatechin, and terpenes as squalene and α-tocopherol. Nitric oxide and ABA stimulated stomatal closure thus increasing water potential. Furthermore, these growth regulators similarly triggered responses associated with vine protection against water stress, as evidenced by increased POD activity, reduced concentration of free AAs as a consequence of less protein degradation, increased concentration of the reducing monosaccharide fructose, and increased accumulation of anthocyanins, such as Peo-3-G, Peo-3-*p* cou and Mal-3-*p* cou. Additionally, differential responses triggered by NO, such as stimulation of APX activity, increase in the concentration of TCA cycle intermediates and the terpenes related to stabilisation and protection of membranes, also suggest differential mechanisms between NO and ABA to counteract the water stress-induced negative effects on vines. However, beyond the NO and ABA induced-responses related to water stress tolerance, there was no growth improvement in the water stressed vines treated with NO and ABA.

The beneficial effects of NO donors in plants have been analysed on diverse plant species subjected to stress and vary depending on the NO donor, NO donor concentration, type of the abiotic stress and the plant species. Furthermore, it is not known if the threshold of NO concentration varies for the different biological actions in different plant organs

(Del Castello et al. 2019). Recently, the positive impacts of NO-releasing nanoparticles have been demonstrated in maize plants under salinity stress, by improving plant growth at lower NO concentration compared to plant treatment with free NO donor (Seabra and Oliveira 2016). Taken together, the administration of NO to vines under water stress conditions represents a promising strategy for use in viticultural production, however, more studies are required in this field.

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Supporting information

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Figure S1. (a) Vitis vinifera cv. Malbec vines during water stress treatment. (b) Daily climatic variables measured inside the greenhouse. (c) Absorbance spectra of polyethylene film covering the greenhouse. Solar photosynthetically active radiation (PAR) inside the greenhouse was 70% of external PAR.