OENO One
VINE AND WINE OPEN ACCESS JOURNAL
By iVES International Viticulture & Enology Society

DOI:10.20870/oeno-one.2022.56.1.5373
OPEN ACCESS

Ozone treatments to induce systemic-acquired resistance in leaves of potted vines: molecular responses and NIR evaluation for identifying effective dose and exposition duration

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Associate editor:
José Miguel Martínez Zapater

Received:
10 January 2022

Accepted:
11 February 2022

Published:
23 February 2022

ABSTRACT

The European Community has recently imposed considerable restrictions on the use of pesticides, with the establishment of a regulatory framework for the sustainable use of agro-chemicals. However, in the viticulture sector, the intensive use of chemical pesticides, as well as sulfur and copper, is often required. Recently, ozone has been proposed as a possible environmentally friendly tool for controlling the development of pests on vines. However, little is known about the parameters linked to the practical application of ozone for controlling grapevine pests and how it triggers plant defence mechanisms. The main aim of this preliminary study was to determine the concentration of ozone and exposure duration in a treatment for stimulating the expression of systemic acquired resistance (SAR)-related genes, without inducing toxic effects and affecting vine health. In the first trial, three different combinations of ozone concentration and duration of treatment were tested on potted grapevines: i) gaseous ozone at 300 ppb for 12 hours, ii) gaseous ozone at 100 ppb for 6 hours, and iii) gaseous ozone at 100 ppb for 3 hours. Based on the results of the first trial, the potted vines were treated with just 100 ppb for 3 hours in a second trial. Leaves at different developmental stages were sampled. The expression level of systemic acquired resistance-related genes was analysed 12 hours and 7 days after each treatment. Furthermore, physiological parameters and NIR spectra were analysed. Ozone induced a transient up-regulation (limited to 12 hours after the treatments) of chitinases, β-1,3-glucanase and glutathione-S-transferase. On the other hand, pathogen-related (PR) genes showed a more persistent over-expression. The ozone treatment selectively affected the stomatal conductance depending on the different ozone concentrations. Detected NIR spectra revealed significant structural changes in ozone-treated plants, especially in leaves exposed to higher concentrations of ozone. These results suggest that ozone is able to transiently stimulate the expression of some resistance-related genes even at low and non-toxic doses for the vine leaves.

KEYWORDS: *Vitis vinifera*, stomatal conductance, gene expression, pathogen control, oxidative stress, NIR

INTRODUCTION

In Europe, although the viticulture sector occupies only 3.3 % of the total cultivated area, the use of chemicals for pest control in wine production accounts for the 65 % of the total chemicals employed in the whole of the agricultural sector (Eurostat, 2007; Blanco-Ward *et al*., 2021). In recent years, both environmental concerns and the regulatory framework based on integrated pest management (IPM) imposed by the European Community have compelled a search for new strategies and approaches for the protection of vine from pests (Bahdra, 2015; Modesti *et al*., 2019). The main goal of the IPM is to replace conventional chemical pesticides with more environmentally friendly products. In this context, some studies have suggested the use of ozone at different grapevine phenological stages (Bhadra, 2015; Modesti *et al*., 2019; Campayo *et al*., 2019; Romeo-Oliván *et al*., 2021). Ozone (O_3) is a strong oxidative gas which is already used in different steps of wine production (e.g., clean-in-place programmes, disinfection of post-harvest grapes and sulphur dioxide-free vinification) (Carbone and Mencarelli, 2015; Bellincontro *et al*., 2017; Mencarelli and Bellincontro, 2018). It has also been reported that applying O_3 to harvested grapes under controlled conditions may have positive effects on grapes and wine quality, with an increase in different phenolic fractions and extractability, antioxidant enzyme activity and volatile terpenoids (Desanctis *et al*., 2015; Carbone and Mencarelli, 2015; Bellincontro *et al*., 2017; Modesti *et al*., 2018).

However, there is still a lack of knowledge regarding the overall effects of O_3 applied to vines. High concentrations of O_3 can be deleterious to plant physiology and can often lead to different types of damage, such as a decrease in photosynthetic activity, premature leaves senescence, chlorophyll degradation, metabolic disorders, visible injuries and a decrease in plant productivity. In grape leaves, the damage caused by high O_3 levels is described as oxidative stipple. The first symptoms are generally small, brown and dot-like lesions on the upper surface of the leaf (Musselman, 1985). Most of these primary lesions become necrotic while retaining the original stipple appearance (Richards *et al*., 1959). All these effects are known to be a result of oxidative-related processes (Feng *et al*., 2008; Heath, 2008; Fuhrer, 2009; Arneth *et al*., 2010; Ainsworth *et al*., 2020). On the other hand, when applied at an adequate and controlled concentration, O_3 can have germicidal effects on plant pathogens. Indeed, O_3 is able to oxidise important pathogen cellular components and thereby reduce their growth (Achen and Yousef, 2001; Tzortzakis *et al*., 2007; Olmez *et al.,* 2009). Additionally, it has been reported that induced oxidative stress leads to a pathogen-attack-like response, which includes the activation of systemic acquired resistance (SAR) (Grulke and Heath, 2020; Conklin and Barth, 2004; Langebartels *et al*., 2002). Once O_3 penetrates the leaves through the stomata, reactive oxygen species (ROS) are produced within the cell (Heath, 2007; Health, 2008; Grulke and Heath, 2020). The production of ROS triggers a pathogen-like response: local programmed cell death (PCD) to avoid the spread of the infection, hypersensitive response (HR) and the subsequent activation of pathogen-related proteins (PR) and other SAR-related genes, such as glutathione S-transferase (GST), chitinases (Chit) and β-1,3 glucanase. All these mechanisms are able to gradually shift the local defence response to a more systemic resistance (Conklin and Barth, 2004; Heath, 2007; Heath, 2008; Grulke and Heath, 2020). This ROS-mediated mechanism is activated by O_3 exposition as well. Considering all the above-mentioned issues and the scarce knowledge about the possible use of O_3 (as ozonated water) for IPM in viticulture, the hypothesis for the present work was that treating potted vines (*Vitis vinifera* cv Sangiovese) with appropriate O_3 concentrations and duration of expositions, can be an effective tool for stimulating SAR without inducing any physiological damage to the plant. Laboratory trials were therefore carried out to study the physiological and molecular responses of grapevine leaves to O_3 applied under controlled conditions (potted plants in a greenhouse).

MATERIALS AND METHODS

1. Ozone treatments

Three-year-old grafted vines (*Vitis vinifera* cv Sangiovese grafted onto 1103 Paulsen) in pots (2.4 L; 13 x 13 x 18 cm) containing a mixture of soil, peat and fine sand (1:1:1, v/v/v) under controlled irrigation (1 L per plant daily) were used for the study. Each year, the plants were of uniform size and at the same physiological development stage. The first trial was performed in 2019. O_3 treatments (three in total repeated on the same plants) were applied once a month (corresponding with BBCH 55, 69 and 77). Twenty plants were placed in a 9 m^3 lit (4000 K white LED, 10 W) room at 10 (± 1) °C with an RH of 70 (± 5) % and treated with: i) gaseous O_3 at 300 ppb for 12 h ii) gaseous O_3 at 100 ppb for 6 h and iii) gaseous O_3 at 100 ppb for 3 h (hereafter referred to as first, second and third treatment), using the O_3 generator A series, which was equipped with an O_3 probe (PC Engineering, Uggiate Trevano, Como, Italy) placed inside the treatment room to maintain a stable O_3 concentration throughout the duration of the treatments. Twenty vines did not receive any O_3 treatment and were kept in a 9 m^3 lit (4000 K white LED, 10 W) room at 10 (± 1) °C with an RH of 70 (± 5) % as the control. The concentrations and duration of ozonation were determined according to the conditions employed in previous studies involving ozonation of wine grapes (Carbone and Mencarelli, 2015). The ozone treatments were performed at 10 °C, taking into account that environmental temperature strongly influences O_3 effectiveness and stability, with more pronounced effects at low temperature (Thanomsub *et al*., 2002; Steenstrup and Floros, 2004; Fan *et al*., 2007). Based on the results of the 2019 trial (absence of visual damage on the leaves), the concentration of 100 ppb for 3 h of exposition was used in 2020. A total of three treatments were again applied, repeated on the same plants once a month (BBHC 55, 69 and 77) as described above. In both years, sampling for the molecular analyses

was carried out 12 h and then 7 d after each treatment. Physiological parameters and NIR spectra were monitored 12 h after each treatment.

2. SAR gene expression analysis by RT-qPCR

To study the possible activation of SAR, the expression of resistance-related genes was determined. Two pathogen-related (*PR1* and *PR6*), two chitinases (*Chit B*, *Chit IV*), glutathione S-transferase (*GST*) and β-1,3 glucanase were selected as SAR marker genes (Heath, 2006). Three leaves (one basal, one median and one apical) were collected from one representative shoot of each vine (20 plants per treatment) 12 h and then 7 d after each treatment. Leaves were randomly split between three different tubes, representing three biological replicates. The sample leaves were immediately frozen in liquid nitrogen and stored at -80 °C for the successive analyses. The frozen leaves were ground to powder using a ceramic mortar and pestle pre-cooled with liquid nitrogen. 100 mg of ground tissue were used for total RNA extraction, using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Italy), including DNA digestion with On-Column DNase I Digestion Set (Sigma-Aldrich, Italy). RNA concentration and purity were determined with Nanodrop 2000 spectrophotometer (Thermo Scientific, Italy), verifying the absorbance ratio of 260/280 nm between 1.8 and 2, and the ratio of 260/230 nm between 1.3 and 2. The integrity of the extracted RNA was checked on a 1 % agarose gel. Reverse transcription of the RNA templates to cDNA was carried out using a 50 ng RNA template and 4 µL of ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich, Italy). DDW (Sigma-Aldrich, Italy) was used to reach a final volume of 20 µL. The PCR conditions were set according to the manufacturer's protocol. Gene-specific primers were designed with the NCBI primer designing tool, based on the mRNA sequences of the target genes from the *Vitis vinifera* genome present in GenBank. The primer couples were run on the NBCI Basic Local Alignment Search Tool (BLAST) in order to verify a specific amplification. The primers were synthesised by Sigma-Aldrich (Italy). Before sample analyses, the amplification efficiency of each couple of primers was determined with a standard curve generated using a serial dilution of representative cDNA mixture. Subsequently, each cDNA dilution was used as a template in real-time qPCR reaction and its Ct was determined. The reaction conditions were set as described below. The Ct values were then plotted against the logarithm of the sample quantity and determined by the dilution performed, excluding Ct values above 35. The efficiency was recovered as percentage. The optimal cDNA dilution for a range of acceptable efficiencies (90-110 %) was found to be 1:5 (Livak and Schmittgen, 2001). The forward and reverse sequences, GenBank Accession, as well as the primer efficiencies, are given in supplementary Table S.1. Sample analyses was performed using the SYBR Green PCR Master Mix (Life Technologies™), with a final reaction volume of 10 µl, running on the CFX Connect RT-qPCR System (BioRad©). The RT-qPCR cycle was set as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of amplification with denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. After the 40 cycles, a melt cycle was performed at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s, to detect possible primer dimers or nonspecific amplification in cDNA samples. PCR reactions were run on all biological replicates and a negative control of the PCR mix was performed in addition to the primers in all qPCR runs. For data analysis, the comparative Ct method described in Livak and Schmittgen, (2001) was used. Expression levels were normalised using the *ubiquitin* (*VvUBC*) housekeeping gene. The relative quantification of each gene tested was calculated using the $2^{-\Delta\Delta}$Ct method.

3. Physiological parameters

Twelve h after each treatment, the physiological parameters described below were analysed on three leaves still attached to five randomly selected plants. Ten readings were taken for each leaf. Chlorophyll content was measured using a chlorophyll concentration meter (MC-100, Apogee instruments, inc.). The obtained Chlorophyll Content Index (CCI) was then converted into the concentration of chlorophyll expressed as µmoles per m^2 of leaf surface using a linear equation specific to *Vitis Vinifera* leaves (Padilla *et al*., 2018). Stomatal conductance was analysed using a leaf porometer (Decagon Devices Leaf Porometer, Decagon Devices, Inc.) equipped with an SC-1 sensor (Decagon Devices, Inc) and expressed in µmol $H_2O/m^{-2}/s$. For the photosynthetic activity measurements, two different parameters, FT and QY, were obtained using a fluorometer (FluorPen FP 110, Photon Systems Instruments, spol. Sr.o.). The leaf surface was first covered with aluminum foil and incubated in the dark for 20 min, then the reading was taken for each leaf. FT indicates the steady-state chlorophyll fluorescence in leaves adapted to darkness and is expressed as relative units. QY is the effective quantum yield of photosystem II and is calculated using the ratio maximal fluorescence intensity to maximal variable fluorescence (Fv/Fm).

4. NIR spectra acquisition

Twelve h after each treatment, five healthy-looking leaves (two basal, two median and one apical), including the three attached ones used for the physiological parameters analyses, from five randomly selected plants, were analszed by detecting NIR (Near Infrared) spectra using a Luminar 5030 Miniature Hand-held NIR Analyzer (Brimrose, Baltimore, MD), based on the NIR-AOTF (Acousto- Optically Tunable Filter) principle (Barnaba *et al*., 2014). Measurements were performed using the diffuse reflectance detection method and raw spectra were detected in transmittance. Detection was conducted in the 1100–2300 nm range, with 2 nm wavelength increments. Raw spectra were manipulated for absorbance (log 1/T) transformation using SNAP! 2.03 software (Brimrose). Ten spectra were taken for each leaf and then averaged to one spectrum per leaf. The obtained 5 spectra per plant were averaged again to one spectrum per plant. The resulting measurements were used for the subsequent chemometric calculations.

5. Statistical analysis

All the aforementioned data (except NIR spectra) were statistically analysed through Shapiro-Wilk and Bartlett's test to test normality and homogeneity of variances. Once these pre-requisites had been established, the data were compared by unpaired T-Test and Tukey's HSD post-hoc test at $p < 0.05$ using Graphpad Prism 7.01 (GraphPad Software, La Jolla, CA, USA) separately for each sampling time and treatment. Both pattern recognition and regression modelling were performed as chemometric approaches on NIR spectra, which had been detected and manipulated as described in Materials and Methods 4. An agglomerative Hierarchical Cluster Analysis (HCA), based on the Ward's method and a principal component analysis (PCA) calculation, were operated on auto-scaled raw spectra, and the resulting clusterisations were graphically reported as a dendrogram. Moreover, Partial Least Square Discriminant Analysis (PLSDA) regressions were carried out with absorbance spectra (log 1/T) as independent variables (X-block), as well as classes of grouped samples as dependent variables (Y-block) (Fordellone *et al*., 2018). The regressive results were reported as score plots, with latent variables (LVs) representing the graph axes; confusion matrices and tables were also calculated for statistical indexes of performance, and robustness for both calibration and prediction models. In both PCA and PLSDA computation, the data were cross-validated using the leave-one-out method (Fearn, 2002; Westerhuis *et al*., 2008). All the computations were performed by Matlab R2013a (MathWorks®, Natick, MA, USA) and PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA).

RESULTS

To investigate the most effective combination of O_3 concentration x duration of exposition, three different treatments were tested in 2019. The first treatment lasting for 12 h with an O_3 concentration of 300 ppb induced serious visible damage to the leaf surfaces (Figure 1A). The second one performed maintaining the vines at 100 ppb of O_3 for 6 h again showed visual foliar damage (Figure 1B). The damage caused by these two treatments (i.e., 300 ppb for 12 h and 100 ppb for 6 h) were already visible by the end of the treatments. The first treatment resulted in burn damage over the entire surface of the leaves; 80-90 % of the total leaves were damaged. Meanwhile, the second treatment induced oxidative stipple disease (Musselman, 1985), which is characterised by brown and dot-like lesions on the upper side of the leaf (Picture 1.B). These stipple burns were visible on half of the leaves of the treated plants. The third treatment of 100 ppb for 3 h did not cause any visible damage (Figure 1C), and therefore this combination was selected for the trial performed in 2020.

1. Gene expression

The gene expression data from the 2019 trials are reported in Figure 2. *Chit IV* (Figure 2A) showed significant higher expression in the leaves of O_3-exposed vines 12 h after the first treatment of 300 ppb for 12 h, while the other treatments did not alter the expression of this gene. On the other hand, *Chit B* (Figure 2B) and *GST* (Figure 2C) were statistically up-regulated in O_3-treated leaves 12 h after the first and the second treatments (300 ppb for 12 h and 100 ppb for 6 h respectively). Finally, *β-1,3 glucanase* (Figure 2D), *PR1* (Figure 2E) and *PR6* (Figure 2F) showed a significantly higher expression in the O_3-treated leaves even with the less impacting third treatment, the only treatment not inducing visible damage. In particular, *PR1* and *PR6* showed a more persistence up-regulation compared to the other genes. Indeed, higher expression levels in the O_3-treated leaves were reached not only 12 h post treatments, but also 7 d after the second and the third treatments in terms of *PR1*, and 7 d after the first and the second treatments in terms of *PR6*.

Based on the observations carried out in 2019, 100 ppb of O_3 for 3 h was the only treatment applied in 2020. As observed in the previous year, the plants treated in 2020 did not show any visible damage (i.e., none of the leaves were damaged). Regarding the expression of SAR-related genes, most of them were up-regulated in treated leaves compared to the control (Figure 3). In detail, *Chit IV,* which in the previous year was not over-expressed with 100 ppb of O_3, was strongly

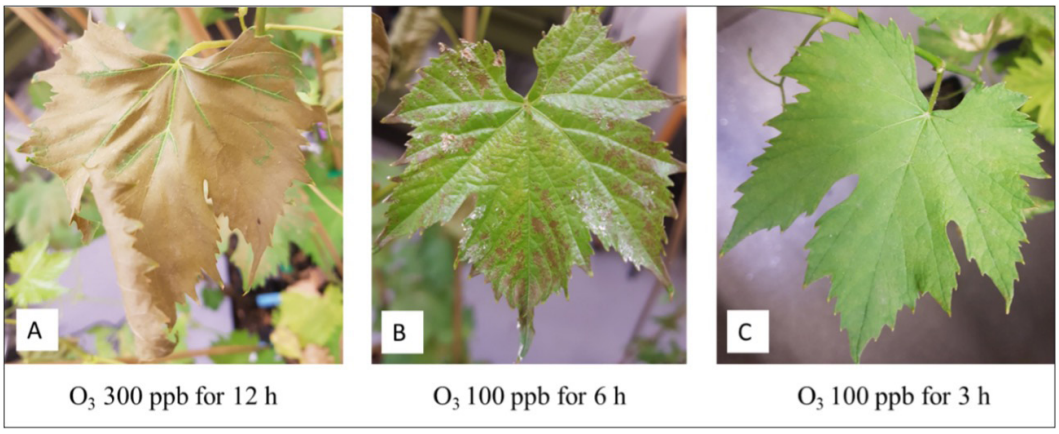

FIGURE 1. Leaf surface of 2019 O3-treated potted vines (cv Sangiovese) at the end of the following treatments: A) 300 ppb of O3 for 12 h, B) 100 ppb of O3 for 6 h, C) 100 ppb of O3 for 3 h.

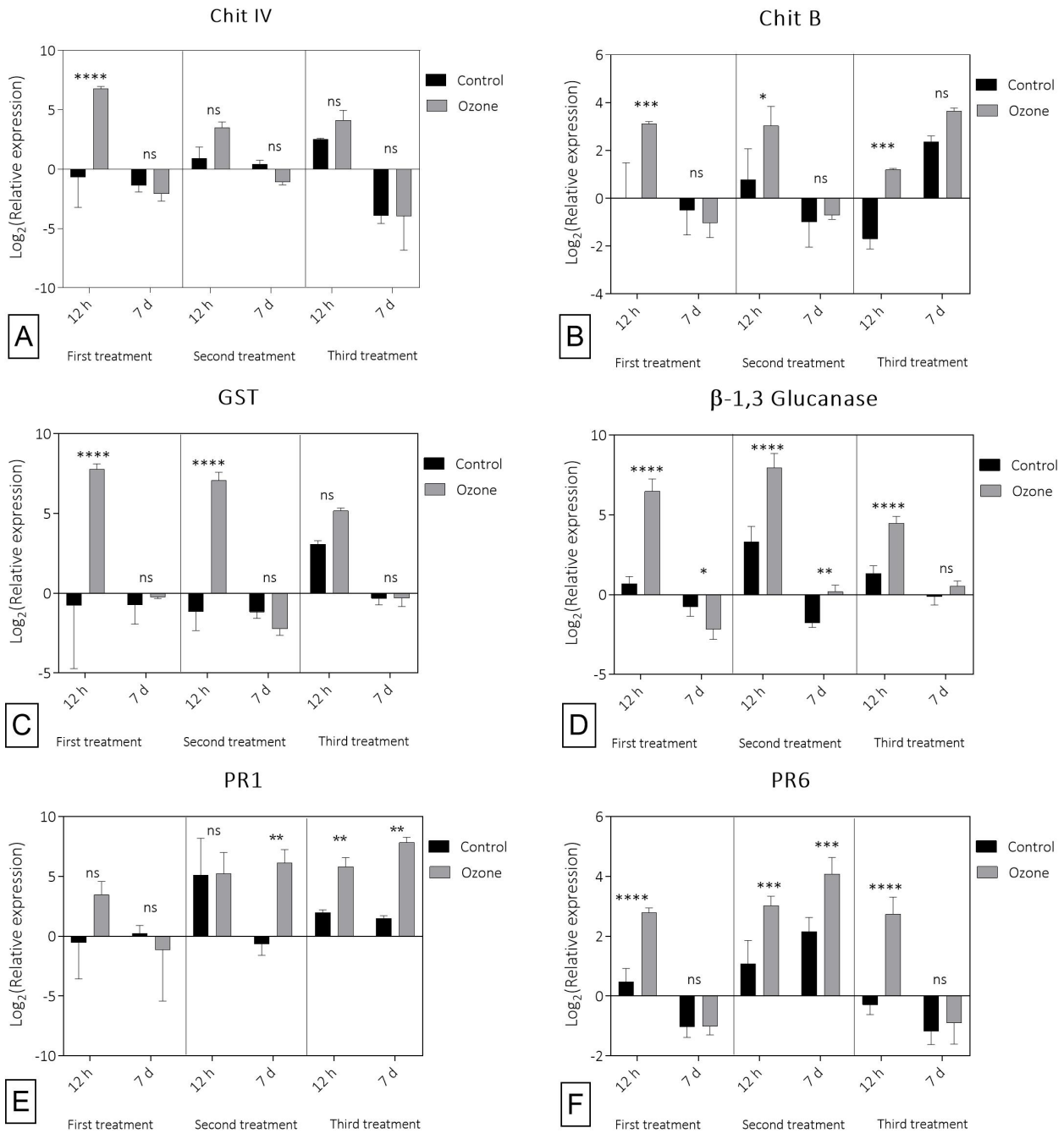

FIGURE 2. Relative expression level of A) Chit IV, B) Chit B, C) GST, D) β-1,3 Glucanase, E) PR1, and F) PR6, analysed by RT-qPCR in leaves collected from 2019 ozone-treated vines at 10 °C (Ozone) and untreated vines at 10 °C (Control), 12 h and 7 d after the following treatments: 300 ppb of O3 for 12 h (First treatment), 100 ppb of O3 for 6 h (Second treatment) and 100 ppb of O3 for 3 h (Third treatment). The average value of three biological replicates is reported with bars representing SD. Stars indicate differences between sample value (****$p < 0.0001$; ***$p < 0.009$; **$p < 0.03$; *$p < 0.01$) based on unpaired T-test performed separately for each sampling time and treatment.

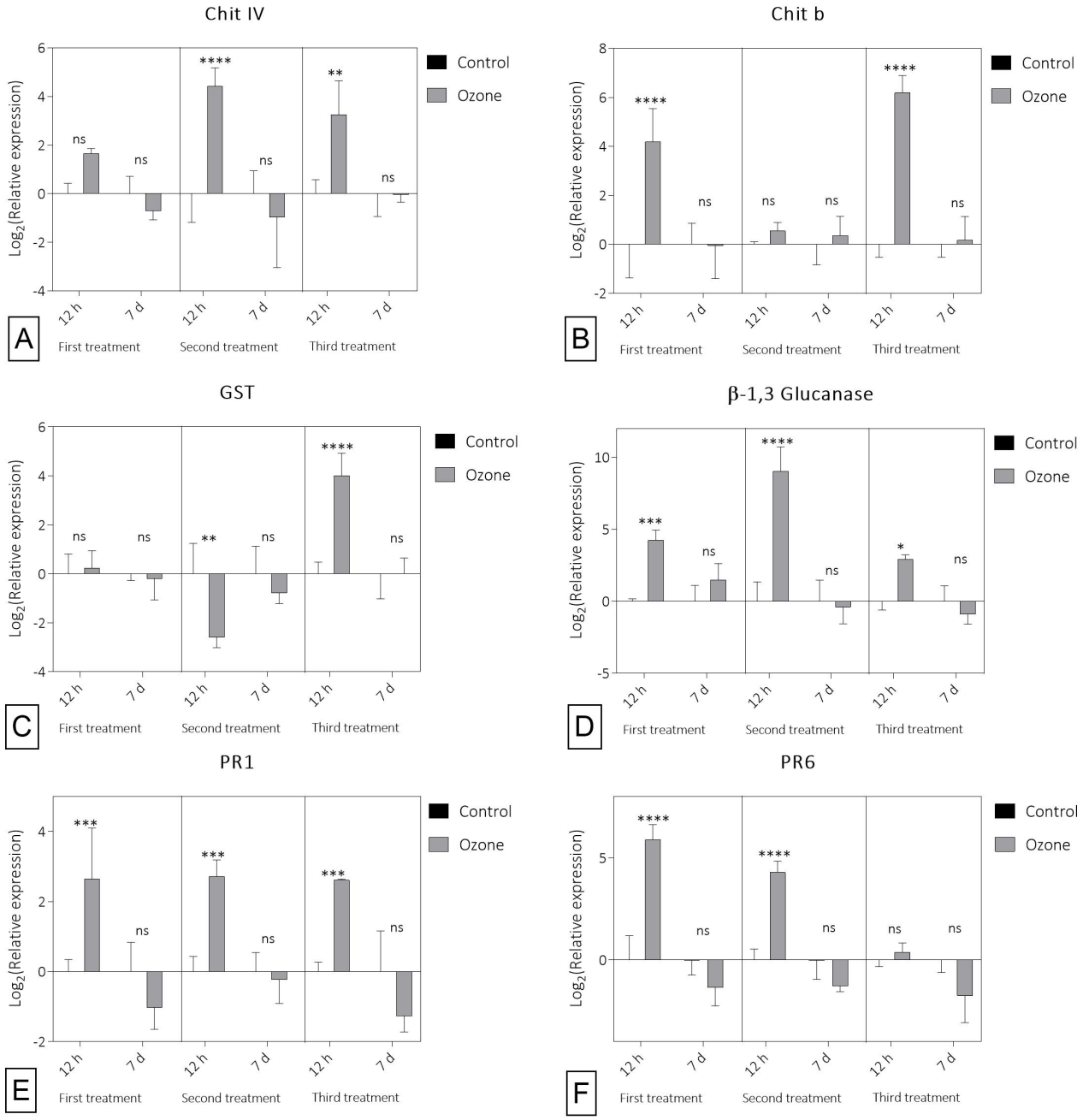

FIGURE 3. Relative expression level of A) Chit IV, B) Chit B, C) GST, D) β-1,3 Glucanase, E) PR1 and F) PR6 analysed by RT-qPCR in leaves collected from 2020 ozone-treated vines at 10 °C (Ozone) and untreated vines kept at 10 °C (Control), 12 h and 7 d after the first, second and third treatment with 100 ppb of O_3 for 3 h. The average value of the three biological replicates is reported with bars representing SD. Stars indicate differences between sample value (****$p < 0.0001$; ***$p < 0.009$; **$p < 0.03$; *$p < 0.01$) based on unpaired T-test performed separately for each sampling time and treatment.

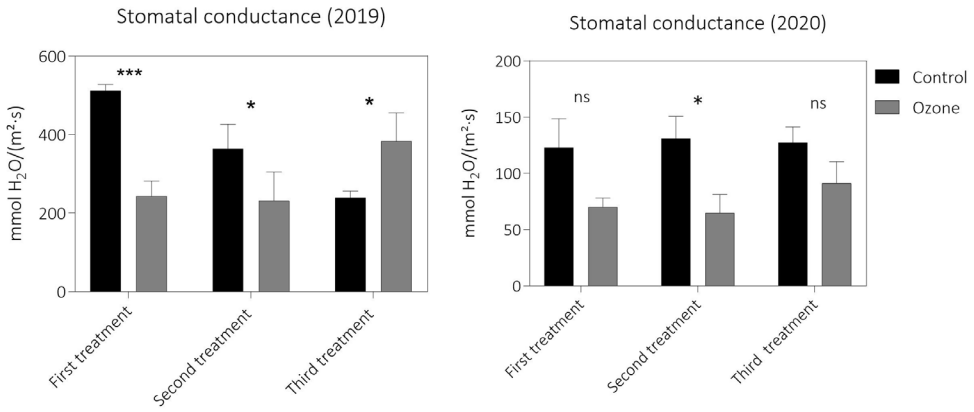

FIGURE 4. Left panel: stomatal conductance expressed in H_2O/m^2 s in ozone treated vines 12 hours after the following treatments: 300 ppb of O3 for 12 hours (first treatment), 100 ppb of O3 for 6 hours (second treatment) and 100 ppb of O3 for 3 hours (third treatment). All the ozone treatment were performed at 10 °C. Control refers to vines kept at 10 °C in normal atmosphere. The average value of fifteen biological replicates is reported with bars representing SD. Stars indicate differences between sample value (***$p < 0.0001$; **$p = 0.008$; *$p = 0.02$) based on unpaired T-test performed separately for each sampling time and treatment. Right panel: stomatal conductance expressed in H_2O/m^2 s in plants 12 hours after each treatment with 100 ppb of O3 for 3 hours at 10 °C (Ozone) and untreated vines kept at 10 °C (Control). The average value of fifteen biological replicates is reported with bars representing SD. Stars indicate differences between sample value (*$p < 0.01$) based on unpaired T-test performed separately between each treatment.

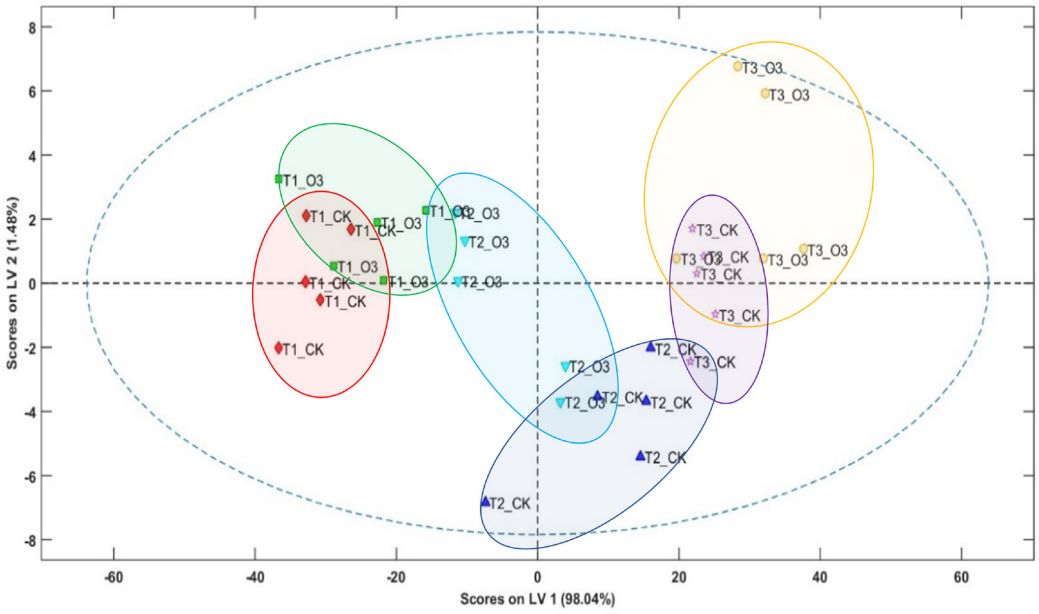

FIGURE 5. PLSDA performed on NIR spectra detected on leaves collected in 2019 12 h after O3 treatment at 10 °C with 300 ppb for 12 h (T1_O3), 100 ppb for 6 h (T2_O3) and 100 ppb for 3 h (T3_O3) or on control leaves (CK) kept at 10 °C for 12 (T1_CK), 6 (T2_CK) or 3 (T3_CK) h. The graph represents the plotting of LV1 versus LV2.

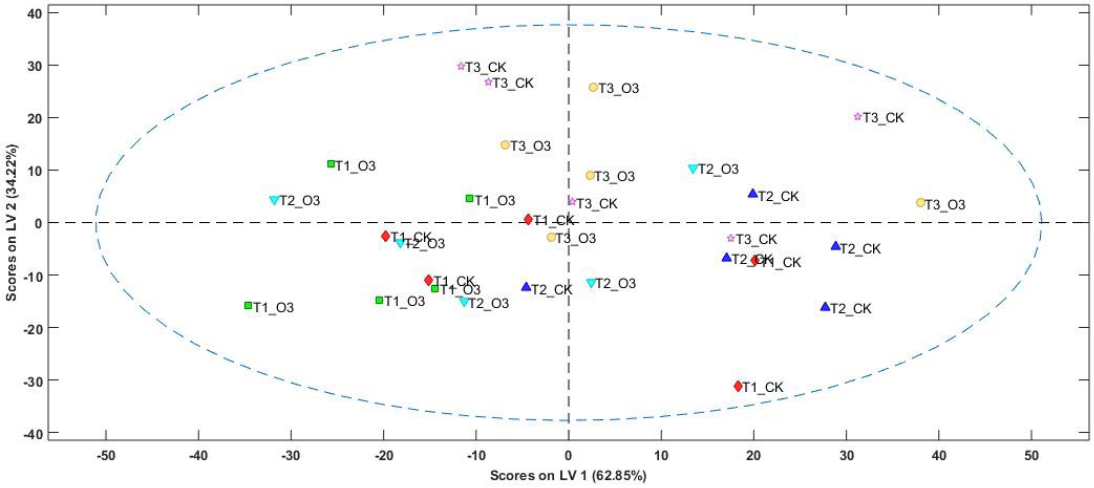

FIGURE 6. PLSDA performed on NIR spectra detected on leaves collected in 2020 trial 12 h after each O3 treatment at 10 °C with 100 ppb for 3 h (T1_O3, T2_O3 and T3_O3), or on control leaves (CK) kept at 10 °C for 3 h (T1_CK, T2_CK, T3_CK). The graph represents the plotting of LV1 versus LV2.

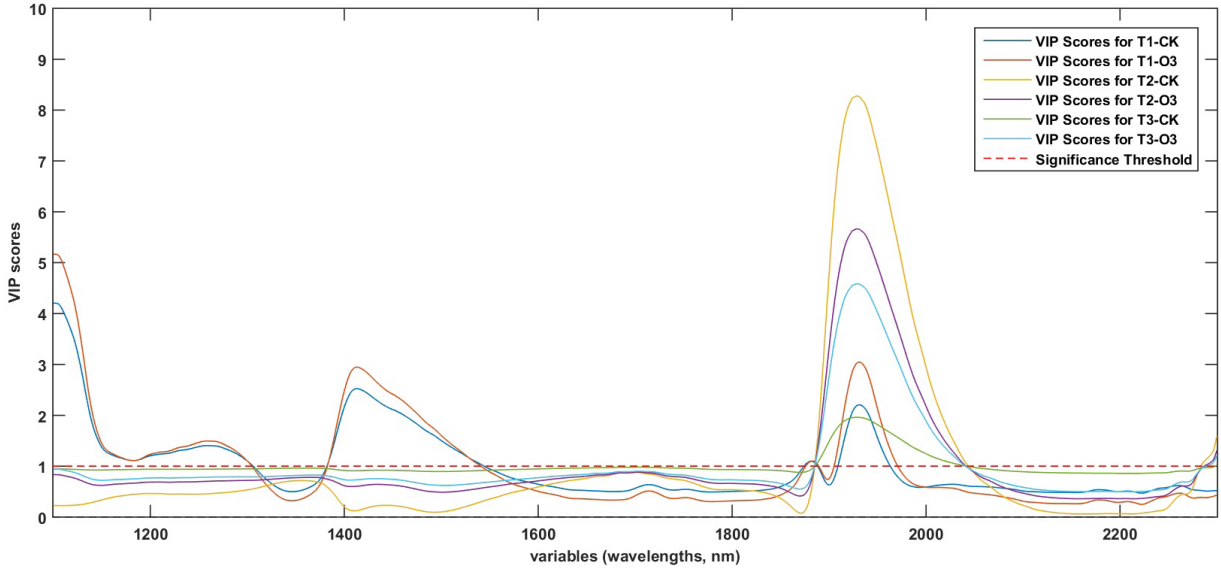

FIGURE 7. variable importance for projection values (VIP) by wavelength for PLSDA-model of 2019 NIR spectra (Figure 6) detected on leaves collected 12 h after O3 treatment at 10 °C with 300 ppb for 12 h (T1_O3), 100 ppb for 6 h (T2_O3) and 100 ppb for 3 h (T3_O3) or on control leaves (CK) kept at 10 °C for 12 (T1_CK), 6 (T2_CK) or 3 (T3_CK) hours.

up-regulated after the second and the third treatments in the 2020 season (Figure 3A). On the other hand, *Chit B* (Figure 3B) showed significant over-expression in O_3-treated leaves for 12 h after the first and the third O_3 treatments. *GST* (Figure 3C) showed very low expression in leaves after the first O_3 exposition, was significantly down-regulated in O_3-treated vines after the second treatment and was significantly up-regulated after the third treatment, which is similar to the observations for other genes. In accordance with the results of the 2019 trials, the treatment with 100 ppb of O_3 for 3 h was effective in stimulating the activity of *β-1,3 glucanase*, *PR1* and *PR6* (Figures 3D, 3E and 3F respectively). *PR1* and *PR6*, which in the previous year showed a more persistent induction, were up-regulated only 12 h after the O_3 expositions.

2. Physiological parameters

Photosynthetic activity did not show any differences between the O_3-treated and control plants. The trials performed in 2019 revealed a slight reduction of chlorophyll content in O_3-treated leaves 12 h after the third treatment (i.e., 8.063 ± 0.2 µmoles/m^2 in O_3-treated vines vs 6.917 ± 0.24 µmoles/m^2 in control

vines) (data not shown). In 2020, just the FT value was affected exclusively 12 h after the second treatment (FT = 4576 ± 410 in O_3-treated vines vs FT = 6068 ± 314 in control vines) (data not shown). Differences were also observed in both years in terms of stomatal conductance. In particular, stomatal conductance was differently affected in the different years and treatments. Indeed, in 2019 (Figure 4, left), O_3-treated leaves showed a marked decrease in stomatal conductance compared to the control after the first and second treatments. In contrast, after the third treatment the leaves of ozonated plants showed a completely opposite trend. In 2020 (Figure 4, right), O_3-treated leaves showed a significantly lower level of stomatal conductance just 12 h after the second treatment.

3. NIR

PLSDA performed on NIR data from the 2019 trails revealed that the first two latent variables (LVs) explained more than 99 % of the residual variance: 98.04 % (LV1) and 1.48 % (LV2) (Figure 5). The different treatments (T1, T2 and T3) can be seen to be well- segregated in different quadrants. A closer look at the different treatments reveals that a good discrimination is also reached in term of samples (control versus O_3), especially in the third treatment (i.e., T3). However, in the PLSDA model performed on NIR data from the second year (2020), such clear clustering of samples and treatments was not observed (Figure 6), even though about 97 % of the variability was explained through the combination of the first two LVs. As a confirmation of this, the results of the confusion matrix for the samples classification by PLSDA in 2019 revealed an average error of 0.02 in calibration, and 0.05 in Cross-Validation (CV) with high correlation (P = precision = total positive/total positive + false positive) ranging between 0.90 and 0.94 (Supplementary Table S.2). On the other hand, in 2020, the performance of the model in assigning samples to the right class of pertinence was worse. In fact, an average error of 0.11 and 0.23 in calibration and cross validation respectively and a very low correlation, especially in CV (P = 0.26), was observed (Supplementary Table S.3). The identified variable importance in projection (VIP) with a score higher than 1 (VIPs) for the 2019 PLSDA model (Figure 7) were associated with spectral wavelengths of around 1450 and 1920 nm. These spectral regions are commonly associated with water content (Seeling *et al*., 2008; Zhang *et al*., 2012).

DISCUSSION

1. Visible injuries and SAR-related responses

Visible injuries on leaves of vascular plants are indicators of both acute and chronic O_3 exposition (Brace *et al*., 1999). It is well known that relatively high concentrations of O_3 in vascular plants such as *Acer circinatum, Fagus sylvatica* and *Vitis vinifera* cause rapid mesophyll cell death, even after a few hours of exposition, and leads to the most common visible foliar symptoms, such as small red or purple dots, burns, bleaching (loss of green colour for chlorophyll degradation and loss of photosynthesis efficiency) and necrosis (Richards *et al*., 1959; Brace *et al*., 1999; Kadinov *et al*., 2017). In the present study, visible damage to leaves caused by the two most extreme (in terms of concentration or exposition duration) O_3 applications (i.e., 300 ppb for 12 h and 100 ppb for 6 hours) were already present at the end of the treatments. The only treatment which did not induce any visible injury was that performed at 100 ppb of concentration for just 3 h of exposition. 100 ppb can be considered a relatively low amount, and similar concentrations of O_3 can be easily reached in the atmosphere. For example, daily typical O_3 concentrations in central Italy range from 80 ppb between 8:00 and 13:00 to 40 ppb from 13:00 onwards in an ordinary day, and can easily increase under particular environmental conditions (Blanco-Ward *et al*., 2021). However, when exposed to O_3 in a controlled experimental environment, plant growth conditions are optimised and the plants often have higher stomatal uptake. This means that the same O_3 level can be more deleterious for plants grown in laboratory conditions due to a higher O_3 uptake, compared to plants growing under more complex and variegated conditions, such as in the field (Grulke and Heath, 2020). In addition, Sangiovese cultivar has been classified as one of the most O_3-sensitive Italian *Vitis vinifera* cultivars (Blanco-Ward *et al*., 2021). It has been well-established that extensive leaf damage itself can induce a pathogenesis-like response mechanism and that O_3-related defence mechanisms are highly similar to those induced by pathogen infections (Grulke and Heath, 2020). Indeed, the over-expression of resistance-related genes after O_3 exposition has been reported in different species (Sharma *et al*., 1996; Heath, 2008). Therefore, O_3 has been studied not only as an air pollutant, but also as an abiotic elicitor for different crops and plants (Sharma *et al*., 1996; Sandermann *et al*., 1998; Heidenreich *et al*., 2006; Modesti *et al*., 2018). To study the possible SAR activation, the expression level of resistance-related genes was determined in the present study. The up-regulation of related resistance genes after O_3 exposition has already been demonstrated (Sharma *et al*., 1996; Langebartels *et al*., 2002) and reviewed by Heath (2008) and Grulke and Heath (2020). Langebartels *et al*. (2002) demonstrated that the biochemical response of tobacco plants to O_3 exposition (*Nicotiana tabacum* L.) was the same as that observed after a pathogen attack. The underlying mechanism seems to be the production of ROS when O_3 penetrates the stomata. Once ROS are produced, a hypersensitive pathogen-like response (HR) is activated (Heath, 2008; Grulke and Heath, 2020). HR is the biochemical mechanism which prevents the local spread of infection. In a first step, HR leads to rapid cell death in the affected area, thus limiting the growth of pathogens. After that, a systemic resistance is slowly activated (Grulke and Heath, 2020). The activation of this mechanism includes the activity of specific genes (Langebartels *et al*., 2002; Heath, 2018; Grulke and Heath, 2020). In plants, a large number of SAR-related genes take part in SAR activation involving two different mechanisms: i) the recognition of virulence products, or ii) direct interaction with the pathogen's biological structure (van Loon *et al*., 2006); in this study, the two genes categories seem to have reacted in slightly different ways. *PR1* and *PR2*

showed more persistent over-expression when the vines were treated with 100 ppb for 6 h, reaching their maximum expression level 7 d after the treatment. Other studies have reported that the *PR1* gene shows an expression peak 5 d after the infection/wounding (Brederode *et al*., 1991). In addition, it is well known that *PR6* proteins are proteinase inhibitors, being highly stable plant tissue defensive proteins (Datta and Muthukrishnan, 1999). Moreover, the gene expression of this family has been correlated with the accumulation of two key systemic resistance hormones: salicylic acid and jasmonic acid (Datta and Muthukrishnan, 1999); this may explain the more persistent over-expression that was recorded in the present study. On the other hand, the genes involved in the cell wall degradation of fungi (*Chit IV, Chit B* and *β-1,3 glucanase*) showed a strong up-regulation in most cases and in both years after O_3 exposition, but the over expression was transient and limited to the 12-hour post-treatment. An important aspect highlighted in some studies (Watanabe *et al*., 2005) is that the expression of these genes is rapidly stimulated within a few hours after O_3 treatment, and then their expression level drops after a day, suggesting that they are more involved in the early and rapid defence response. The two-year results show that the *GST* gene, which is involved in the detoxification of foreign compounds (Watanabe *et al*., 2005), seems to be the least susceptible gene and 100 ppb of O_3 for 3 h of exposition seems to be insufficient for the up-regulation of the expression of this gene. Taken together, these data indicate that the transient activation of molecular mechanisms resembling the pathogen-induced responses are present in O_3 -treated grapevine leaves.

2. Physiological response

As far as the physiological response to O_3 is concerned, a well-known physiological shift that plants make to "survive" the O_3 effect is stomatal closure, thus limiting O_3 uptake (Grulke and Heath, 2020). Here, the different stomatal behaviour observed in the two years is not completely surprising and might be the result of a number of factors. In fact, stomatal closure, density and dimension are strongly influenced by air humidity, temperature, daily sunlight exposition, internal CO_2 and metabolic state (Turcsanyi *et al*., 2000; Grulke and Heath, 2020), as well as O_3 uptake (Hetherington and Woodward, 2003). Many studies have reported a decrease in stomatal conductance in *Vitis vinifera* after O_3 exposition, thus reducing its uptake (Pellegrini *et al*., 2015; Valletta *et al*., 2016; Geng *et al*., 2017). The results obtained in 2019 after the first and the second treatments, along with the trend observed in 2020, all confirm this behaviour. In contrast, the data obtained for the last treatment in 2019, after which the plants had higher stomatal conductance levels, refers to plants which havehad already been exposed to higher O_3 concentrations for longer (i.e., first treatment of 300 ppb of O_3 for 12 h and second treatment of 100 ppb of O_3 for 6 h). Previous studies have reported that short term (few hours) exposure to low or moderate levels of O_3 is generally associated with a rapid reduction in stomatal conductance. On the other hand, longer exposition to higher levels of O_3 is translated into a sluggish stomatal response (Grulke and Heath, 2020). The induced sluggish response leads to an inefficient control of water loss, because the stomata will remain opened in undesirable environmental conditions and partially closed in external optimal conditions for accumulation (Patterson and Rundel, 1993). This could be the case of the already O_3 stressed plants in 2019. In addition, after long and high-dose O_3 exposition, the sluggish stomatal response is often persistent and leads to an incomplete closure during the night (Barnes *et al*., 1990). Interestingly, the contradictory results obtained in stomatal conductance suggest that leaf physiological response to O_3 is strongly dependent on the time and the concentration of exposition. Another important consideration is related to the regressive model, performed on the NIR spectra, in the discriminating control and O_3-exposed leaves. In 2019, the plants were exposed to higher concentrations and for a longer time which was probably the reason for the good discrimination obtained between spectra from the control and O_3-treated leaves. This observation seems to be confirmed by the fact that, in 2020, leaves exposed to lower concentrations for a shorter time were not so different from their controls. This is of particular interest, considering the NIR spectra ability to give significant indications about internal changes occurring as a consequence of molecular modifications, even related to stress effects (Khaled *et al*., 2018). Furthermore, the clear separation obtained in 2019 after the different treatments (T1, T2 and T3) suggests that NIR spectra was affected - as a consequence of a vibrational response of the molecular overtones - by not only the different O_3 concentrations but also the different treatment durations. Indeed, in 2019, the treatments were based on different exposition time (12, 6 and 3 h); therefore, the control plants were also kept at 10 °C for 12, 6 and 3 h in the first, second and third treatments respectively, and a good segregation among the different treatments is observed.On the other hand, in 2020, all three treatments were the same in terms of duration (3 hours), and it is difficult to differentiate them.

3. Structural response

Lastly, our NIR spectra findings seem to agree with the stomatal behaviour data: in 2019, the plants were more stressed, and stomatal conductance was found to have been affected by each treatment. The induced stomatal response leads to significant modifications in gas exchanges and, consequently, in water content (Patterson and Rundel, 1993). Hence, the identified VIP of the 2019 PLSDA model were associated with the spectral regions commonly combined with water content (Seeling *et al*., 2008; Zhang *et al*., 2012); the ability of the model to discriminate samples from spectra is therefore likely due to the sensitivity of spectra to water regulations that are influenced by stomatal behaviour, as already stated in other published work (Marchica *et al*., 2019). A prolonged and uncontrolled exposure of vines to O_3 has been previously found to often lead to negative and deleterious effects (Pellegrini *et al*., 2015; Valletta *et al*., 2016;

Blanco-Ward *et al.*, 2021); however, the exposition to adequate O_3 concentrations for a shorter time and under controlled conditions can be a useful tool for taking advantage of the O_3-induced oxidative stress (Tonelli *et al*., 2015; Pellegrini *et al*., 2018; Modesti *et al*., 2019; Marchica *et al*., 2019). In the present study, this seems to be confirmed by the fact that in 2020 the plants were exposed to lower concentrations of O_3 for a shorter duration, and therefore no significant alterations occurred in the O_3-treated leaves, even though the SAR-related genes were still up-regulated.

CONCLUSIONS

Ozone treatments induced a temporary up-regulation of most of the systemic acquired resistance-related genes. The remarkable effect of the O_3 treatments differed depending on exposition level. After being subjected to prolonged treatments comprising high levels of O_3, the plants showed serious visible foliar damage, as well as an inefficient control of gaseous exchange due to a sluggish stomatal closure response. This set of data suggests that the higher the O_3 concentration, the higher the possibility for the plant to develop signs of damage, thus demonstrating a strong relationship between plant physiological response and O_3 accumulation. This observation is confirmed by the NIR spectra, which highlight the potential physiological and structural modifications mainly due to changes in water content. On the other hand, the induced oxidative stress and subsequent activation of a pathogen-like response were detected in both years, regardless of the dose and treatment duration. This indicates that when plants are in need of a more systemic defense mechanism, SAR-related processes are always activated. This study provides innovative results for the possible practical application of O_3 on vines, revealing that the combination of 100 ppb for 3 h is effective in stimulating the expression of the SAR-related gene, even when plants remain healthy, as in the case of the control plants. Further investigations will be carried out to highlight the effect of O_3 treatment (gaseous or ozonated water) on other processes/compounds related to SAR activation (such as ROS, salicylic and jasmonic acid biosynthesis) and on the direct oxidative effect on pathogen structure for the control of specific grapevine pathogens.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Vivai Cooperativi Rauscedo (Rauscedo, PN, Italy) and Aziende Vivaistiche Gini (Cenaia, PI, Italy) for supplying the potted plants.

This work has been carried out as part of the networking activities "Oxygen sensing a novel mean for biology and technology of fruit quality" (CA18210), implemented under the COST Action "RoxyCOST" and funded by the European Cooperation in Science & Technology (2019–2023).

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