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CHANGES IN STILBENE COMPOSITION DURING POSTHARVEST OZONE TREATMENT OF 'MOSCATO BIANCO' WINEGRAPES

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ABSTRACT

Stilbenes, including *trans*-resveratrol and its derivatives, are compounds naturally present in grapes and have gained a growing interest due to reported health-promoting properties. The production of resveratrol-enriched table grapes has promoted recent research on stress-induced synthesis of stilbenes. The oxidizing properties of ozone have been successfully exploited to its use as sanitizing agent and stilbene elicitor during table grapes storage. In winegrapes, this study represents the first research focused on the effect of postharvest ozone treatments on the accumulation of stilbene compounds. The study was carried out on Moscato bianco winegrapes (*Vitis vinifera* L.) and several gaseous ozone treatments were investigated differing in ozone dose (30 and 60 µL/L), exposure time (24 h, 48 h, and several days until 30% of weight loss), and delay time until processing (just after and several days after treatment). The stilbene production induced by ozone exposure was assessed in fresh and partially dehydrated winegrapes up to 5, 10, 15, 20, and 30% of weight loss aiming to evaluate the single and combined effect of oxidative and osmotic stresses. The results obtained showed that short-term exposure of fresh winegrapes at 60 µL/L of ozone for 48 h was not effective in inducing resveratrol accumulation just after treatment, but it had an elicitor effect on total stilbenes (+36%) in grapes subsequently dehydrated up to 20% of weight loss with a significant overproduction of *trans*-resveratrol and *trans*-piaceatannol. In addition, long-term and continuous treatments under ozone-enriched atmosphere can be also used during dehydration to sanitize winegrapes without affecting negatively the concentration of stilbenes. Therefore, the use of gaseous ozone during storage and dehydration could be indicated to reduce the use of sulfur dioxide and, depending on ozone dose and exposure time, the synthesis of stilbene compounds could increase.

Keywords: ozone, postharvest treatment, partial dehydration, stilbene compounds, *trans*-resveratrol, winegrapes

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a non-flavonoid phenolic compound that has gained importance as a consequence of its reported beneficial attributes to human health (Hurley, Akinfiresoye, Kalejaiye, & Tizabi, 2014; Kodali et al., 2015; Simão et al., 2012; Witte, Kerti, Margulies, & Flöel, 2014). It is well-known that resveratrol is a scavenger of free radicals and therefore it plays a key role in the prevention of oxidative stress (Truong, Jun, & Jeong, 2018). For this reason, research studies have been recently developed in this field in order to understand its protective action mechanism and molecular targets (Fei et al., 2018). Other stilbenes, such as *trans*-piceide (*trans*-resveratrol-3-O-β-D-glucopyranoside), *trans*-piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) and viniferins, have similar properties.

The increasingly numerous applications of resveratrol and other stilbenes for therapeutic uses and as an ingredient in dietary supplements and functional foods have promoted studies based on engineering yeast for high level production of these compounds (Li, Schneider, Kristensen, Borodina, & Nielsen, 2016). However, some plants can synthetize naturally resveratrol, this phenolic compound being found in several fruit berries such as raspberries, mulberries, blueberries, and grape berries (Flamini, Mattivi, De Rosso, Arapitsas, & Bavaresco, 2013). Particularly, grape skins are considered one of the main dietary sources of this stilbene, although its concentration is rather low, genotype-dependent and influenced by ripening, environmental conditions, cultural practices, and growing season (Cantos, Tomás-Barberán, Martínez, & Espín, 2003; Gatto et al., 2008; Jeandet, Bessis, & Gautheron, 1991; Versari, Parpinello, Tornielli, Ferrarini, & Giulivo, 2001; Vincenzi et al., 2013). With the purpose of exploiting this natural source of stilbenes, the overexpression of the resveratrol biosynthesis pathway could be induced through biotic and/or abiotic stress. In fact, grapevine and even postharvest grapes react against injuries, pathogens attack, and several abiotic stress conditions (physical, mechanical, or chemical) by synthetizing stilbene compounds as a natural defense mechanism (Douillet-Breuil, Jeandet, Adrian, & Bessis, 1999; Hasan & Bae, 2017).

UV-C irradiation and ozone exposure are the most important abiotic elicitors that can enhance the synthesis and accumulation of stilbenes in table and wine grapes. Increased stilbene concentrations have been stimulated as a response to UV-C irradiation of pre-harvest (Jeandet et al., 1991) and postharvest grapes (Cantos, Espín, & Tomás-Barberán, 2002; Cantos, García-Viguera, de Pascual-Teresa, & Tomás-Barberán, 2000; Cantos et al., 2003). Instead, the oxidative stress associated with the gaseous ozone exposure has been only exploited in postharvest grapes (Artés-Hernández, Artés, & Tomás-Barberán, 2003; Sarig et al., 1996). Nevertheless, the stilbenes biosynthesis in postharvest table grapes after gaseous ozone treatment was comparable (Sarig et al., 1996) or more elicited with respect to UV-C irradiation (González-Barrio et al., 2006). Short-term ozone treatments of white table grapes induced the increase of stilbenes biosynthesis after 2 days of storage at 22 ºC (González-Barrio et al., 2006). In red table grapes, shock ozone treatment during 38 days of cold storage caused an overproduction of stilbene compounds, particularly *trans*-resveratrol (Artés-Hernández et al., 2003).

In wine industry, the strong oxidant activity of ozone can be used for reducing the growth of spoilage microorganisms present on the berry surface in fresh winegrapes and even during postharvest withering (Botondi et al., 2015; Cravero et al., 2018; Guzzon, Franciosi, Moser, Carafa, & Larcher, 2018). This aspect is particularly important for the production of special wines, particularly fortified, *sfursat*, and *passito*, from partially dehydrated grapes. Therefore, the effectiveness of ozone for sanitizing purposes, leaving no residues, makes it possible its use as an alternative to sulfur derivatives (Bellincontro, Catelli, Cotarella, & Mencarelli, 2017). Recent studies have reported the advantages of using a grapevine-shoot stilbene extract (Vineatrol®) to preserve the quality of SO_2 free red wines, particularly regarding phenolic composition, color intensity, and sensory characteristics (Raposo et al., 2018). Consequently, the benefits of winegrapes exposure to gaseous ozone as sanitizing treatment could be increased exploiting the advantages of ozone-induced stress to elicit stilbene compounds. Taking into account that postharvest treatments with gaseous ozone have induced the synthesis of stilbenes in table grapes,

the aim of this study was to evaluate the grape stilbene response to continuous ozone treatment during both postharvest storage and withering process in order to produce stilbene-enriched winegrapes. For this aim, i) the impact of long-term ozone treatments was assessed at different stages of grape dehydration until reaching the 30% of weight loss necessary for the production of *passito* wines, and ii) the effect of ozone concentration and exposure time during short-term treatments was evaluated both in fresh grapes just after treatment and in grapes subsequently dehydrated at 20% of weight loss, which are used to produce special dry wines such as fortified. This study reports for the first time the combined effect of grape ozone exposure and partial dehydration on stilbene induction, as well as the effectiveness of postharvest ozone-induced stress on the accumulation of stilbenes in winegrapes.

2. Materials and methods

2.1. Ozone treatment and dehydration process of winegrapes

Moscato bianco (*Vitis vinifera* L.) white winegrapes were harvested in a commercial vineyard located in the Piedmont wine region (Asti province, North-West Italy, 44°43' N, 8°10' E) in 2015. Two independent experiments were carried out. For the first one, about 1 kg of grape berries were randomly selected for fresh grape analysis (P0, without postharvest air and ozone exposure). Afterwards, about 20 kg of grape berries were submitted to long-term treatments (dehydration process) as follows. Small clusters of 4-5 berries were arranged in a single layer into ten perforated plastic boxes (30 cm × 20 cm, about 2 kg of grape berries per box) for correct aeration and then withered into thermo-hygrometrically controlled chambers. The use of small clusters instead of whole bunches allows to better assure a representative sampling during the dehydration process. Five boxes of small clusters were partially dehydrated under air atmosphere (AR, control samples) and the other five boxes were dehydrated under ozone-enriched (30 µL/L; Río Segade et al., 2017) atmosphere (OZ, treated samples). The dehydration process until reaching

30% of berry weight loss was completed in 24 days for control and ozone-treated grapes. About 1 kg of AR and OZ grape samples were randomly taken at 5, 10, 15, 20, and 30% of weight loss (P5, P10, P15, P20, and P30, respectively) for partially dehydrated grape analysis after long-term and continuous ozone and air exposure.

For the second experiment relative to short-term treatments, another 20 kg of small clusters consisting of 4-5 berries were distributed in a single layer into ten perforated plastic boxes (30 cm × 20 cm, about 2 kg of grape berries per box). The first two sets of fresh grape berries were only exposed to atmospheric air for 48 h (control samples). Four ozone treatments were carried out using two sets of fresh grape berries for each: ND24, ozone exposure for 24 h with a normal dose of ozone (30 µL/L; Río Segade et al., 2018) and then air exposure for 24 h; ND48, ozone exposure for 48 h at 30 µL/L; HD24, ozone exposure for 24 h with a high dose of ozone (60 µL/L; Río Segade et al., 2018) and then air exposure for 24 h; and HD48, ozone exposure for 48 h at 60 µL/L. One of the two sets resulting from each test was used for short-term stored grape analysis (control-S, ND24-S, ND48-S, HD24-S, and HD48-S). The other five sets were then partially dehydrated into thermo-hygrometrically controlled chambers during 13 days, until reaching a weight loss of 20%. The samples obtained were used for partially dehydrated grape analysis after short-term ozone and air exposure (control-D, ND24-D, ND48-D, HD24-D, and HD48-D).

During all the treatments, a data logger (HOBO H8 RH/Temp, Onset Computer Corporation, Bourne, MA) continuously monitored and recorded the thermo-hygrometric conditions. The short- and long-term treatments under air or ozone-enriched atmosphere were carried out at 20 ± 2 ºC and 60 ± 5% of relative humidity (Río Segade et al., 2017; Río Segade et al., 2018). An ozone generator (C32-AG, Industrie De Nora Spa, Milan, Italy) with a nominal production capacity of 32 g O_3/h, equipped with a BMT 964 UV-photometric ozone analyzer (BMT Messtechnik Gmbh, DE) controlling the ozone generator output, was used for continuous exposure of grape berries to ozone-enriched air at 120 m^3/h flow (Río Segade et al., 2017; Río Segade et al., 2018).

2.2. Standard chemical parameters determination

Three replicates of 100 fresh grape berries were randomly selected and each replicate was manually crushed. The grape juice obtained was centrifuged and then used for determining total soluble solids content (ºBrix, as SSC) with an Atago 0–32 ºBrix temperature compensating refractometer (Atago Corporation, Tokyo, Japan).

2.3. Extraction and determination of stilbene compounds

For each sample, about 200 berries were immediately frozen and stored at −20 °C until analysis. Then, three sub-samples of 40 berries were randomly selected. For each replicate, the skins were manually removed from the frozen berries, weighed, and freeze-dried. The berry skins were treated following the method reported by Sun, Ribes, Leandro, Belchior, and Spranger (2006) and by Vincenzi et al. (2013), with some modifications. Briefly, one gram of freeze-dried skins was introduced into 40 mL of methanol containing 250 µL of *trans*-hydroxyl stilbene as internal standard (200 µg/mL in ethanol) and 50 µL of hydrochloric acid. After homogenization for 1 min with Ultraturrax (IKA Labortechnik, Staufen, Germany), the berry skin samples were stirred for 48 h in closed containers at room temperature in the dark. The methanol extract containing polyphenols was obtained by centrifugation at 5000 × g for 5 min and the solid residue was washed twice with 5 mL of methanol. The washing solutions were recovered and added to the first supernatant, the mixture being then filtered with a 0.2 µm PTFE syringe filter (Advantec, Milano, Italy). After adding 2 mL of water, the extract was almost completely evaporated to dryness at 35 °C under vacuum. The residue obtained was suspended in 20 mL of water and stilbene compounds were extracted twice with 10 mL of ethyl acetate for 15 min. The ethyl acetate fraction containing stilbenes was recovered carefully, dried using anhydrous sodium sulfate, filtered through a Whatman 589/3 paper (Maidstone, United Kingdom), and evaporated to dryness at 35 °C under vacuum. The residue was dissolved in 2 mL of a solution containing methanol and 50 mM of

formic acid (1:1) and then centrifuged at 14000 × g for 10 min. All organic solvents and acids were purchased from Carlo Erba (Milano, Italy).

Stilbenes were separated in a C_{18} LiChrospher column (250 mm × 4 mm, 5 μm, Agilent Technologies, Milano, Italy) using a HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector (Waters Corporation), according to Vincenzi et al. (2013). The mobile phase consisted of 50 mM of formic acid (solvent A) and methanol (solvent B), working at 1.0 mL/min of flow-rate. A gradient program was used, increasing from 0 to 10% of B in 3 min, from 10 to 30% of B in 5 min, from 30 to 44% of B in 35 min, from 44 to 55% of B in 2 min, from 55 to 75% of B in 15 min, and from 75 to 100% of B in 1 min. After washing with solvent B for 2 min, the column was re-equilibrated with solvent A. The injection volume was 20 μL and the column temperature was set to 40 °C. *Trans*- and *cis*-isomers were detected at 306 and 285 nm, respectively. Stilbene identification was performed by comparing the retention time of each compound with that of the respective commercially available standard of *trans*-piceid, *trans*-piceatannol, *trans*-resveratrol, *trans*-hydroxystilbene, and *ε*-viniferin (Extrasynthese, Genay Cedex, France). *Cis*-isomers were obtained by exposure of the corresponding *trans*-molecules to UV-light for 1 min and the extinction coefficient of the *trans*-forms was assumed for their quantification. Individual stilbenes were quantified using external calibration based on the peak area. The results for stilbene concentrations were expressed as μg/g of skins (fresh weight, FW) to better compare the different treatments and to reduce the effect of berry weight loss due to dehydration.

2.4. Statistical analysis

All data were statistically treated using the XLStat-Pro software from Addinsoft (Paris, France). Two-way analysis of variance (ANOVA) and the HSD Tukey's test for $p < 0.05$ were used to analyze significant differences. Three replicates were analyzed for each sample. In the first experiment, treatment (air or ozone) and berry weight loss (0, 5, 10, 15, 20 and 30%) were the two

main factors while ozone dose (30 and 60 μL/L) and exposure time (24 and 48 h) were in the second experiment.

3. Results and discussion

3.1. Total stilbenes

Most of works published on stilbene compounds of winegrapes are referred to red varieties. In untreated Moscato bianco winegrapes (27.0 ± 0.2 °Brix), the total concentration of stilbenes found in our work (8.21 μg/g of berry FW) agreed with the values reported for some red winegrape varieties ranging from 1.97 μg/g of berry FW for Cabernet sauvignon to 15.43 μg/g of berry FW for Merlot (Cantos et al., 2003).

To our knowledge, this is the first study concerning the effect of ozone exposure on stilbenes in winegrape varieties. **Figure 1** shows the effect of different ozone treatments on the total concentration of stilbenes in Moscato bianco winegrapes. The first issue was to evaluate if exposure time under ozone-enriched atmosphere during long-term treatment, particularly postharvest dehydration under controlled thermohygrometric conditions, affects significantly these compounds in relation to postharvest dehydration under air atmosphere. As can be observed in **Figure 1a**, ozone exposure caused a significant decrease in total concentration of stilbenes at the beginning and the end of withering when a berry weight loss of 5, 20, and 30% was reached (ANOVA, $p < 0.05$). For intermediate exposure times, and therefore intermediate dehydration, ozone-treated winegrapes had a slightly lower concentration of total stilbenes than air-treated samples at the same value of weight loss. In addition, no significant effect was observed during winegrape dehydration under air atmosphere but the lowest total concentrations of stilbenes were found for 5% of weight loss (reduction of –11.8% in relation to fresh sample). The same trend was observed for ozone-exposed winegrapes, although the decrease observed at the beginning of dehydration (5% of weight loss) was significant achieving –39.8% in relation to fresh sample

(HSD Tukey's test, $p < 0.05$). These results highlight that a first metabolic response to abiotic stress (decrease of total stilbenes) occurred at 5% of berry weight loss due to osmotic stress in control samples and to the combined effect of osmotic and ozone stress in treated grapes. A second stress response (increasing total stilbenes) was observed beyond 20% of weight loss in control samples and 10% of weight loss in ozone-exposed grapes because of the berry resilience to the first stress. Metabolic changes were also observed in other studies during grape dehydration influencing volatile compounds (Costantini, Bellincontro, De Santis, Botondi, & Mencarelli, 2006). Both treatment and time effects were significant (ANOVA, $p < 0.001$ and 0.05, respectively) however a lack of interaction occurred.

Taking into account that no elicitor effect of ozone was observed on stilbenes for long-term treatments when an ozone concentration of 30 µL/L was used, which induces the overproduction of volatile compounds in the same winegrape variety as reported in a previous study (Río Segade et al., 2017), we also investigated if the grape berries respond differently to the oxidative stress by ozone exposure during short-term treatments. Particularly, the effect of ozone dose and exposure time on total stilbenes was studied in fresh winegrapes just after short-term ozone treatment and even in grapes subjected to the same ozone exposure and then partially dehydrated at 20% of weight loss. **Figure 1b** shows that, in short-term stored fresh winegrapes, the total concentration of stilbenes decreased in all ozone-treated samples in relation to control (air-treated for 48 h), this reduction being only significant (HSD Tukey's test, $p < 0.05$) in ND48 and HD24 samples (about –30%). In fact, the ozone treatment at the highest dose (60 µL/L) and the longest time (48 h) limited this decrease with respect to control up to –8.9%. In fresh winegrapes, the ozone dose and exposure time effects were not significant, but the interaction between them was significant on the total concentration of stilbenes (ANOVA, $p < 0.01$). In withered winegrapes (**Figure 1b**), it is important to evidence that short-term ozone treatment made it possible to reduce the significant decrease in total stilbenes (ANOVA, $p < 0.05$) caused by the partial dehydration at 20% of berry weight loss (from –29.8% in withered control to –3.4%, –19.8%, and +5.2% in ND24, HD24, and

HD48 samples, respectively) or even to overturn it significantly in ND48 samples (+17.2%; ANOVA, $p < 0.01$). When ozone-treated samples were compared to control for withered winegrapes, the total concentration of stilbenes in ozone-exposed samples was slightly higher, with the exception of HD24 sample. Although in this case the differences were not significant due to the high variability of the results obtained, the richest samples in stilbenes were HD48 showing an increase of +36.5% in relation to withered control. Therefore, the ozone dose effect was not significant whereas the exposure time effect and interaction dose × time was on the total concentration of stilbenes in withered winegrapes (ANOVA, $p < 0.05$ for both). These last results agreed with the increased concentration of total stilbenes, reported by Artés-Hernández et al. (2003) in Napoleon red table grapes shock-treated with 8 ppm of ozone for 30 min every 2.5 h, up to 58% after 38 days of cold storage at 0 °C and up to 2-fold its concentration after additional 6 days of shelf-life at 15 °C for a cumulative weight loss of 4.3%.

3.2. Stilbene composition

As observed for total stilbenes, the concentrations of individual stilbene compounds in untreated Moscato bianco winegrapes, such as *trans*-resveratrol (6.09 µg/g of berry FW), *trans*-piceid (0.56 µg/g of berry FW), *trans*-piceatannol (0.92 µg/g of berry FW), and *ε*-viniferin (0.64 µg/g of berry FW), were within the range reported by Cantos et al. (2003) for red winegrape varieties (from 0.47 for Cabernet sauvignon to 8.97 for Merlot, from 0.19 for Tempranillo to 1.69 for Monastrell, from 0.19 for Monastrell to 1.75 for Merlot, from 0.13 for Cabernet sauvignon to 3.62 for Monastrell, respectively for resveratrol, piceid, piceatannol, and total viniferins, all expressed as µg/g of berry FW). Furthermore, Vincenzi et al. (2013) also evidenced a high variability in Italian red winegrape varieties for the concentrations of *trans*-resveratrol (from 19 to 508 µg/g of skin FW), *trans*-piceid (from undetected to 1196 µg/g of skin FW), and *trans*-piceatannol (from undetected to 72.1 µg/g of skin FW). **Table 1** shows that the concentrations of individual stilbene compounds in untreated Moscato bianco winegrapes (P0 samples) were within

the range reported by Vincenzi et al. (2013). Regarding white winegrapes, the concentrations found in our study for Moscato bianco agreed with those observed in native Romanian varieties for *trans*-resveratrol (from 41.8 to 74.7 µg/g of skin FW), *trans*-piceid (from undetected to 9.1 µg/g of skin FW), and *trans*-piceatannol (from undetected to 66.4 µg/g of skin FW) (Urcan et al., 2016). In the present study, *trans*-resveratrol was the major stilbene compound (47–74% from the total concentration) whereas *cis*-isomers of stilbenes were not detected in control and ozone-treated samples. This confirms that *cis*-isomers are not synthesized in grapes under water and ozone stress conditions. With the aim of knowing if the variations observed in total stilbenes are due to one individual stilbene compound or to the combination of individual effects, the influence of long-term and short-term ozone treatments was also assessed on the stilbene composition.

Table 1 shows the impact of long-term ozone treatment during postharvest winegrapes dehydration on stilbene composition. When air- and ozone-treated winegrapes were compared for each sampling point during postharvest dehydration, ozone exposure affected significantly the concentration of all the stilbene compounds determined for at least one level of dehydration (P samples), with the exception of *trans*-resveratrol. Significantly lower concentrations of *trans*-piceid and *trans*-piceatannol were observed at the beginning (P5, 5% of berry weight loss) and the end of the dehydration process (P20 and P30 for *trans*-piceid, and P30 for *trans*-piceatannol) for ozone-exposed samples (ANOVA, $p < 0.05$) whereas this same behavior was evidenced for ε-viniferin (oligomeric form) at P5, P10, and P20 samples (ANOVA, $p < 0.05$, 0.05, and 0.001, respectively).

The dehydration effect was not significant for *trans*-piceatannol in relation to fresh winegrapes. Instead, the concentration of *trans*-resveratrol was significantly lower at any sampling point with respect to fresh sample, particularly for P5 and P10 samples dehydrated under both air and ozone-enriched atmosphere (HSD Tukey's test, $p < 0.05$). During postharvest dehydration of grape berries, the effectiveness of stress-induced stilbene synthesis is strongly influenced by time and temperature parameters, as confirmed by Versari et al. (2001) through the stilbene synthase

(STS) activity. Particularly, these authors evidenced a steady increase of *trans*-resveratrol in Corvina red winegrapes during the withering process, and in particular the maximum accumulation occurred at 74th day of traditional withering (ambient temperature in a naturally ventilated room) when grape berries reached about 20% of weight loss, and then there was a decline. In our study, instead, a decrease of *trans*-resveratrol concentration was observed at the beginning of grape dehydration, probably due to the different withering conditions (20 ºC and 60% of relative humidity) or to the different response of the Moscato bianco variety to osmotic stress with respect to Corvina red variety. In any case, after the initial fall, even in our study *trans*-resveratrol increased during the withering process, reaching the highest accumulation at 15% of weight loss, and its concentration decreased slightly thereafter. Resveratrol synthesis occurs via the phenylalanine pathway, where phenylalanine ammonia lyase (PAL), coumaroyl-CoA ligase (4CL), cinnamate-4-hydroxylase (C4H), and STS play a key role (Hasan & Bae, 2017). Another important aspect to consider is that also 4-coumaroyl-CoA is a precursor for the flavonoids biosynthesis and therefore STS competes with chalcone synthase (CHS) for the same substrates (Flamini et al., 2013). This competition between STS and CHS could reduce the resveratrol accumulation during dehydration, when total concentration of flavonoids is increasing (Rolle et al., 2013).

Regarding *trans*-piceid and *ε*-viniferin, the concentration increased significantly during the dehydration process under air (HSD Tukey's test, $p < 0.05$), as also *ε*-viniferin did in ozone-treated samples (HSD Tukey's test, $p < 0.05$). However, *trans*-piceid showed an irregular trend under ozone exposure reaching the highest concentration in P10 samples. The above mentioned increases were higher than those corresponding to berry dehydration and therefore the synthesis of *trans*-piceid and *ε*-viniferin could occur. The interaction treatment × dehydration was significant only for *trans*-piceatannol and *ε*-viniferin (ANOVA, $p < 0.05$ and 0.01, respectively). González-Barrio et al. (2006) highlighted that the accumulation of stilbene derivatives is induced sequentially under abiotic stresses (gaseous ozone exposure), the monomeric form of resveratrol being biosynthesized

first, followed by dehydrodimers (*ε*-viniferin and *δ*-viniferin) and then dehydrotrimers. In the present study, the initial decrease of *trans*-resveratrol concentration during dehydration, particularly at 10% of berry weight loss, could be justified by the induced accumulation of *trans*-piceid and *ε*-viniferin. The subsequent increase in *trans*-resveratrol concentration until reaching the maximum value in P15 samples, which remained practically unchanged for longer dehydration, seems to be due to a berry response against the greater osmotic stress (prevalent and significant dehydration effect according to **Table 1**; ANOVA, $p < 0.05$).

The effect of ozone dose and exposure time for short-term treatment was also evaluated on stilbene composition in fresh Moscato bianco winegrapes just after treatment and also in grapes partially dehydrated at 20% of weight loss after ozone treatment (**Table 2**). In short-term stored fresh winegrapes, the results obtained for *trans*-resveratrol agreed with those found for total stilbenes, for which the concentration was significantly lower in ND48 and HD24 samples than in control samples (HSD Tukey's test, $p < 0.05$). The dose effect (ANOVA, $p < 0.05$) prevailed on the time effect (ANOVA, $p > 0.05$) on *trans*-resveratrol, although the interaction dose × time was more significant (ANOVA, $p < 0.01$). The dose effect (ANOVA, $p < 0.01$) also prevailed on the time effect (ANOVA, $p > 0.05$) for *trans*-piceid. However, no ozone-treated sample showed *trans*-piceid, *trans*-piceatannol, and *ε*-viniferin concentrations significantly different to control samples even though the highest values corresponded to the longest treatment with the highest ozone dose (HD48 samples).

In this study, the ozone effect was more relevant for stilbenes induction when the grape berries were short-term treated and then partially dehydrated until 20% of weight loss. In withered winegrapes (**Table 2**), ozone-treated samples before dehydration showed an increased concentration of *trans*-resveratrol and *trans*-piceatannol, with the exception of HD24 sample, as previously observed for the concentration of total stilbenes. When compared to control samples, significantly higher concentrations of *trans*-resveratrol were found in ND24 and HD48 samples whereas those of *trans*-piceatannol corresponded to ND48 and HD48 samples (HSD Tukey's test,

$p < 0.05$). Therefore, the interaction dose × time was significant for *trans*-resveratrol (ANOVA, $p < 0.01$) but the time effect prevailed for *trans*-piceatannol (ANOVA, $p < 0.01$) on the dose effect (ANOVA, $p < 0.05$). Thereby, ozone induced the accumulation of stilbene derivatives different from resveratrol. Particularly, the highest concentrations of *trans*-resveratrol found in samples ozone-treated for 24 h at the normal dose (ND24) and in samples ozone-treated for 48 h at the high dose (HD48) agreed with the trend observed previously in *Vitis* species, for which resveratrol biosynthesis has two peaks: the first one corresponding to an initial defensive response to rapidly counteract stress whereas the second one being a more lasting protective response to prevent new stresses (Douillet-Breuil et al., 1999). In addition, stilbene synthase mRNAs have two maxima related to the differential expression of different genes (Wiese, Vornam, Krause, & Kindl, 1994).

For all ozone treatments, *trans*-piceid concentration remained constant or slightly increased with respect to control withered samples, and the time effect prevailed (ANOVA, $p < 0.01$) on the dose effect (ANOVA, $p > 0.05$) as occurred for *trans*-piceatannol. In Napoleon red table grapes shock-treated with 8 ppm of ozone lasting 30 min every 2.5 h, Artés-Hernández et al. (2003) reported an induced synthesis of resveratrol and piceid after 38 days of storage at 0 °C followed by 6 days of shelf life at 15 °C. In our study, the increases observed were lower probably because the experiment was performed at usual winery temperature (20 °C instead of 15 °C). Heath (2008) highlighted that different metabolic pathways are stimulated during ozone treatments depending on dose or exposure time regimes.

4. Conclusions

The use of ozone as sanitizing agent and stress-inductor of resveratrol synthesis during table grapes storage has been largely discussed. Nevertheless, stilbene compounds have been scarcely quantified in winegrapes and there is no work published to date on stress-induced resveratrol production by ozone exposure of winegrapes. Therefore, the present study provides important information on the impact of ozone treatments differing in dose and time on the

concentration of stilbene compounds in postharvest fresh and dehydrated winegrapes. When ozone was used for sanitizing purposes during short-term storage of fresh postharvest winegrapes, the normal dose tested (30 µL/L of ozone) for 24 h or the high dose (60 µL/L of ozone) for 48 h were recommended to avoid significant losses of total or individual stilbene derivatives, probably due to the activation of other metabolic pathways. Instead, controlled applications of this oxidative stressor during short-term treatments can lead to a relevant induction when subsequent grape withering is performed. In fact, short-term treatments with high ozone dose (60 µL/L) and long time exposure (48 h) not only prevented the loss of stilbene compounds caused by the dehydration process, but also induced their accumulation. In this case, the elicitor effect of ozone was particularly significant for *trans*-resveratrol (+37.3%) and *trans*-piceatannol (+41.9%). Unexpectedly, long-term and continuous ozone treatments have not stress-induced *trans*-resveratrol production in winegrapes, even though gaseous ozone could be considered an important tool to avoid mycobiota spoilage on winegrapes during dehydration without negatively and significantly affecting the concentration of this stilbene. Therefore, the use of gaseous ozone could have important applications in winemaking in order to produce wines with high added-value taking into account both the possible reduction of SO_2 addition and, under certain conditions, increased contents of health-promoting stilbenes.

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FIGURE CAPTIONS

Figure 1. Total stilbene content (µg/g of skin fresh weight) in ozone-treated Moscato bianco grapes: (a) at harvest and during postharvest dehydration under air and ozone-enriched atmosphere (30 µL/L), and (b) in fresh and withered samples after short-term treatment with ozone at different exposure doses and times. All data are expressed as average value and error bars correspond to standard deviations (n=3). Different lower and capital Latin letters indicate significant differences among dehydration levels for long-term ozone-treated samples (a) and among short-term treatments for fresh grapes, respectively, according to the HSD Tukey-b test ($p < 0.05$). Asterisks denote significant differences between air and ozone treated samples at the same weight loss during dehydration (a) and between fresh and withered samples for each short-term treatment (b): *, ** and ns indicate significant differences according to ANOVA at $p < 0.05$, 0.01 and not significant, respectively. ND24, ozone at 30 µL/L for 24 h; ND48, ozone at 30 µL/L for 48 h; HD24, ozone at 60 µL/L for 24 h; and HD48, ozone at 60 µL/L for 48 h.

Table 1. Stilbene composition (µg/g of skin fresh weight) of Moscato bianco grapes long-term treated with ozone and air during postharvest dehydration.

Sample	*trans*-Resveratrol	*trans*-Piceid	*trans*-Piceatannol	*ε*-Viniferin
P0	43.29±5.46b,β	4.01±0.79a,αβ	6.57±1.43αβ	4.52±0.34a,α
P5-AR	27.84±3.79a	4.84±0.82ab	8.24±1.40	7.22±0.93b
P5-OZ	22.51±3.59α	2.91±0.15α	5.12±0.42α	4.61±0.44α
Sign.	ns	*	*	*
P10-AR	26.36±4.95a	8.89±1.29bc	8.53±0.71	8.31±0.80bc
P10-OZ	24.19±2.78α	8.93±0.37γ	8.78±1.11β	5.64±0.24αβ
Sign.	ns	ns	ns	*
P15-AR	37.33±5.36ab	8.39±2.87abc	6.83±1.39	6.77±1.33ab
P15-OZ	30.95±2.33α	6.28±0.66β	7.19±0.46αβ	6.67±1.08β
Sign.	ns	ns	ns	ns
P20-AR	30.50±5.65ab	9.50±0.19bc	8.01±0.17	11.33±0.32d
P20-OZ	27.41±3.89α	5.22±1.09αβ	7.55±0.81αβ	6.38±0.32β
Sign.	ns	*	ns	***
P30-AR	30.23±3.33ab	12.22±0.40c	10.13±0.06	10.22±1.22cd
P30-OZ	25.55±7.46α	5.44±1.59αβ	5.71±1.35α	8.96±0.69γ
Sign.	ns	*	*	ns
Sign. AR	*	**	ns	***
Sign. OZ	**	**	*	***
Treatment	ns	**	**	***
Dehydration	*	***	ns	***
Treatment × *Dehydration*	ns	ns	*	**

All data are expressed as average value ± standard deviation (n = 3). *Sign.*: *, **, *** and ns indicate significant differences according to ANOVA at $p < 0.05$, 0.01, 0.001 and not significant, respectively, between air (AR) and ozone (OZ, 30 µL/L) treatments at the same weight loss. Different Latin letters within the same column indicate significant differences among dehydration levels for the air treatment according to the HSD Tukey-b test ($p < 0.05$). Different Greek letters within the same column indicate significant differences among dehydration levels for the ozone treatment according to the HSD Tukey-b test ($p < 0.05$). P: sampling point at a defined berry weight loss (0, 5, 10, 15, 20, and 30%).

Table 2. Stilbene composition (µg/g of skin fresh weight) of fresh and withered Moscato bianco grapes after short-term treatment with ozone at different exposure doses and times.

Sample	***trans*-Resveratrol**	***trans*-Piceid**	***trans*- Piceatannol**	**ε-Viniferin**
Control-S	41.97±2.38b	10.33±1.47ab	7.52±1.11	6.19±0.64
Control-D	23.22±0.81α	7.74±2.13αβ	6.78±0.31α	10.33±1.47
Sign.	***	ns	ns	ns
ND24-S	39.07±2.28b	7.64±2.08ab	7.33±0.05	5.32±0.72
ND24-D	35.22±1.35β	9.03±1.71αβ	8.70±1.13αβ	12.24±1.04
Sign.	ns	ns	ns	*
ND48-S	27.10±2.80a	6.65±1.49a	7.13±0.07	5.05±0.07
ND48-D	21.99±0.12α	13.01±0.90β	9.98±0.60β	8.89±0.12
Sign.	ns	**	**	***
HD24-S	25.47±2.74a	10.08±0.06ab	7.83±1.33	5.95±0.04
HD24-D	17.71±0.91α	6.47±0.21α	6.12±0.48α	7.79±1.62
Sign.	*	**	ns	ns
HD48-S	33.54±4.58ab	11.88±1.60b	9.09±0.13	6.25±1.06
HD48-D	31.89±5.82β	12.66±3.33β	9.62±1.43β	9.08±0.88
Sign.	ns	ns	ns	ns
Sign. S	**	*	ns	ns
Dose	*	**	ns	ns
Time	ns	ns	ns	ns
Dose × *Time*	**	ns	ns	ns
Sign. D	**	*	*	ns
Dose	ns	ns	*	*
Time	ns	**	**	ns
Dose × *Time*	**	ns	ns	ns

All data are expressed as average value ± standard deviation (n = 3). *Sign.*: *, **, *** and ns indicate significant differences according to ANOVA at $p < 0.05$, 0.01, 0.001 and not significant, respectively, between stored fresh (S, just after postharvest ozone treatment) and withered grapes (D, grapes dehydrated at 20% of berry weight loss) for the same treatment. Different Latin letters within the same column indicate significant differences among treatments for fresh grapes according to the HSD Tukey-b test ($p < 0.05$). Different Greek letters within the same column indicate significant differences among treatments for withered grapes according to the HSD Tukey-b test ($p < 0.05$). ND24, 30 µL/L of ozone for 24 h; ND48, 30 µL/L of ozone for 48 h; HD24, 60 µL/L of ozone for 24 h; and HD48, 60 µL/L of ozone for 48 h.

Highlights

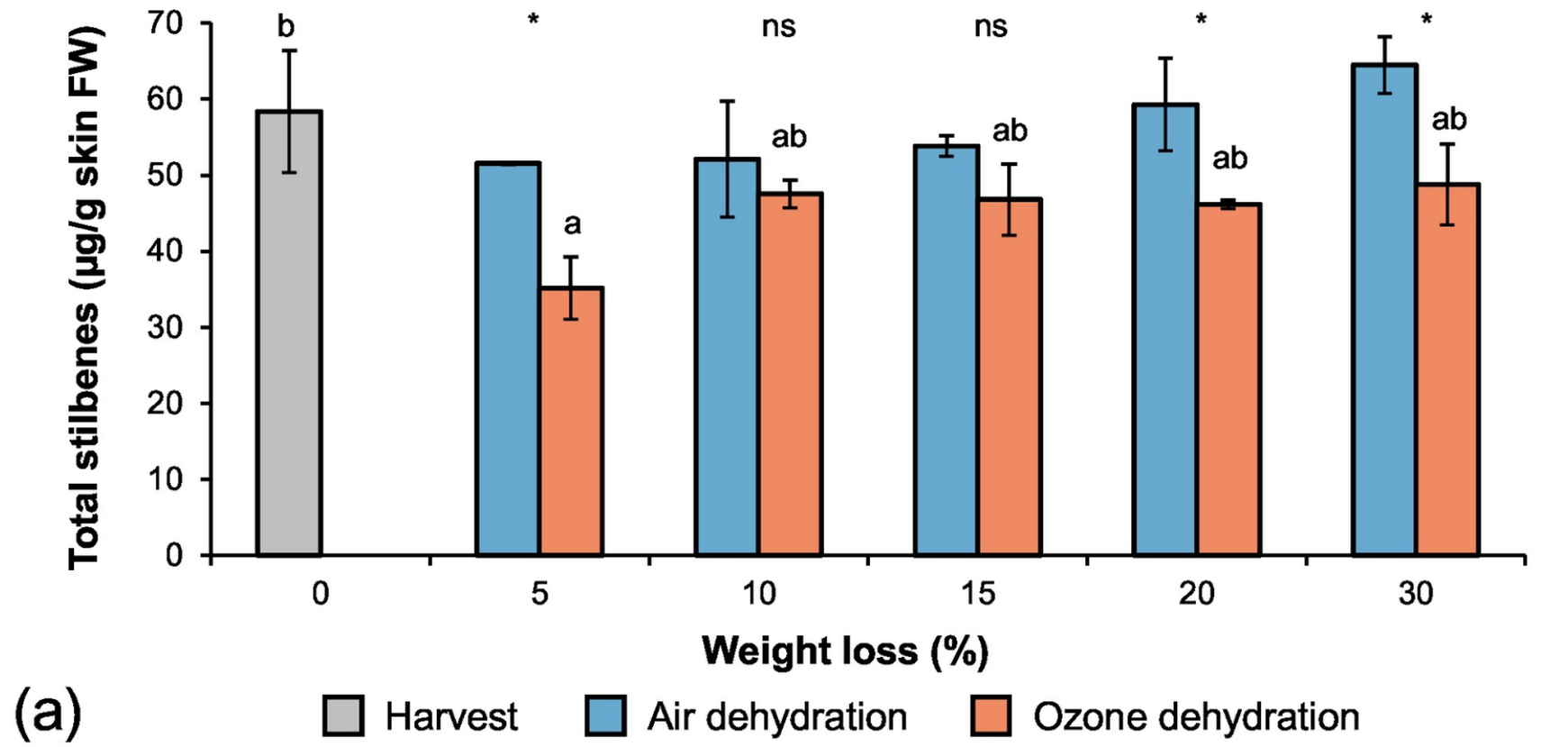
-Effect of postharvest ozone treatment on stilbene synthesis was studied in winegrapes

-Several treatments were investigated differing in ozone dose and exposure time

-Single and combined effects of ozone and dehydration stresses were also assessed

-Ozone exposure at 60 µL/L for 48 h was effective to induce stilbenes after withering

-Stilbene concentration was not affected negatively during long-term ozone treatments


Total stilbenes (µg/g skin FW)
70
60
50
40
30
20
10
0
b
*
ns
ns
*
*
a
ab
ab
ab
0
5
10
15
20
30
Weight loss (%)
(a)
Harvest
Air dehydration
Ozone dehydration

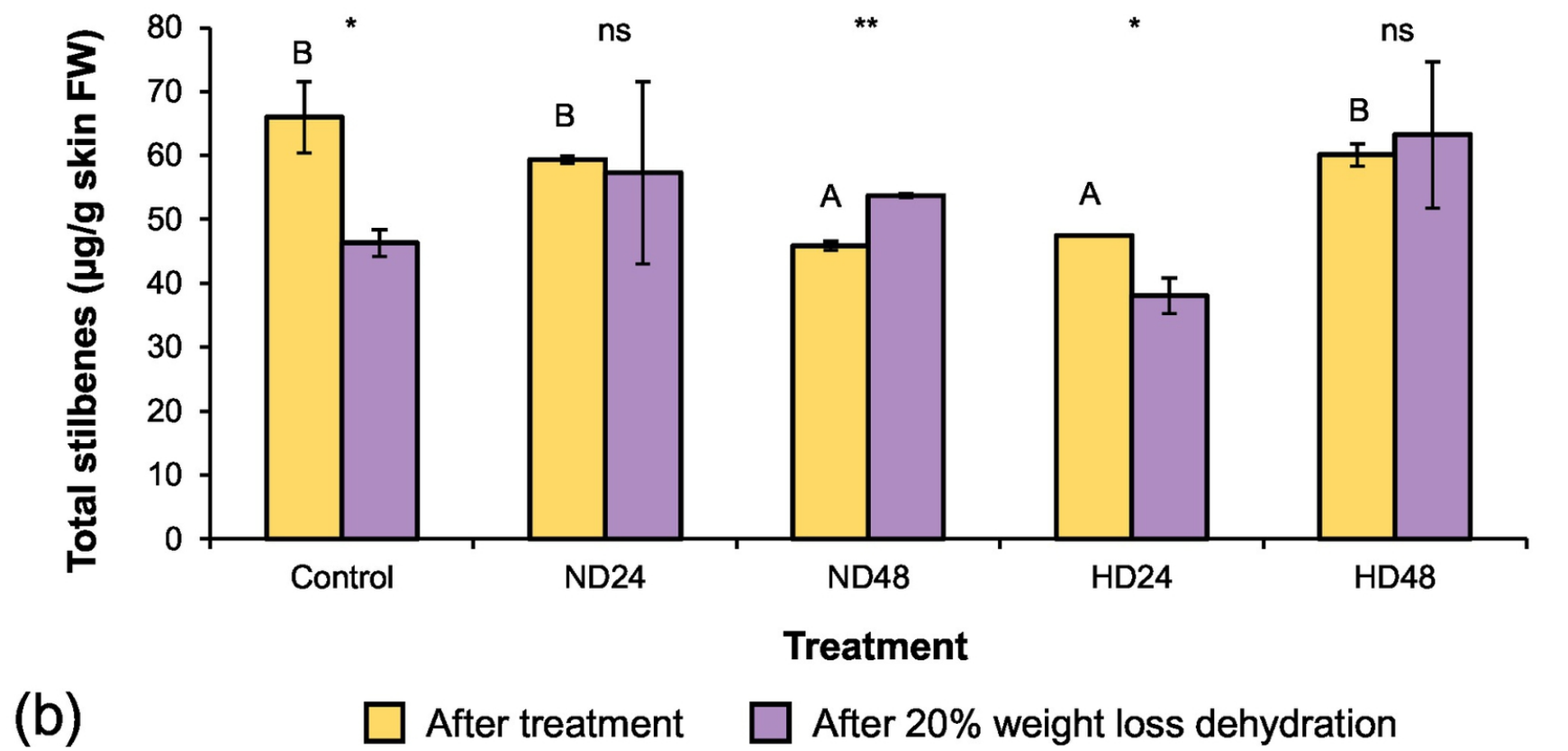

Total stilbenes (µg/g skin FW)
80
70
60
50
40
30
20
10
0
B
*
B
ns
A
**
A
*
B
ns
Control
ND24
ND48
HD24
HD48
Treatment
(b)
After treatment
After 20% weight loss dehydration

Figure 1