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Original article

Oxidative enzymes and functional quality of minimally processed grape berries sanitised with ozonated water

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Summary Thompson Seedless (TS) and Black (BS) grapes sanitised with 2, 4, 6, 8 mg L^{-1} O_3 or NaOCl (100 mg L^{-1}) were stored 21 days at 5 °C. Ozonated water stimulated the respiration rate, especially after 5 days of storage, and increased superoxide dismutase and catalase activity compared to NaOCl-sanitised grapes. Total polyphenol content (TPC) was 23–50% higher in TS and 18.5–28% higher in BS samples sanitised with ozonated water. Twofold higher total antioxidant capacity (TAC) was registered in TS at all of the evaluated O_3 doses while the doses of 6 and 8 mg L^{-1} increased TAC by 19–30% in BS. The use of ozonated water as a sanitising method, especially at 6 and 8 mg L^{-1} doses, improved the functional quality and maintained low microbial counts on fresh-cut grapes being a good alternative for the industry.

Keywords Antioxidant activity, antioxidant enzymes, grapes, ozone, phenolic compounds.

Introduction

Different fruit, including table grapes, can be used as raw materials for minimally processed elaboration. The use of grapes in the minimally processed fruit industry is advantageous because processing involves rachis elimination, allowing an increase in berry shelf-life period because the deterioration of grapes is mainly associated with rachis browning (Del Nobile *et al.*, 2009). Rachis browning is considered the second most important problem in table grapes causing consumer rejection and fruit waste (Lichter, 2016).

Washing and sanitation are considered critical processes for the minimally processed fruit industry as, at this step, it is possible to remove or inactivate pathogens and microorganisms that cause deterioration, ensuring food safety (Delaquis *et al.*, 2004). Chlorine derivatives are the most widely used, but some of them are associated with the production of potentially carcinogenic compounds such as trihalomethanes (Bao Loan *et al.*, 2016). Additionally, the washing step requires a large amount of cold wastewater with very high levels of biological oxygen demand (Ölmez & Kretzschmar, 2009). Among the sanitation method alternatives to chlorine derivatives, ozone (O_3) has gained considerable importance. This strong oxidising agent can be used as a gas or dissolved in water to delay microbial growth or fungal decay on whole products, including grapes (Silveira *et al.*, 2010; Feliziani *et al.*, 2014; Glowacz *et al.*, 2015). In addition, due to its oxidising nature, it exerts effects on the physiological behaviour of the living tissue (Alothman *et al.*, 2010). The oxidative stress in plant cells occurs due to high leakage of electrons towards O_2 during photosynthetic and respiratory processes, leading to enhanced reactive oxygen species (ROS) generation (Sánchez-Rodríguez *et al.*, 2012).

ROS, such as superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^{-}) and singlet oxygen ($^1O_2$), damage cellular structures and macromolecules by directly attacking lipid membranes, inactivating metabolic enzymes and damaging nucleic acids, leading the cell to die (Mittler, 2002; Gill & Tuteja, 2010; Murshed *et al.*, 2013). Therefore, an increase in ROS in vegetal tissue due to a stress condition determines the activation of enzymatic and nonenzymatic antioxidant systems (Jacobo-Velázquez *et al.*, 2011).

The enzymatic antioxidant system includes superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and

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glutathione reductase (GR; EC 1.6.4.2). The superoxide radical (O_2^-) is dismutated to H_2O_2 by SOD, and CAT, APX and GPX metabolise H_2O_2 to H_2O (Martínez-Hernández *et al.*, 2013). The nonenzymatic antioxidant system includes polyphenols and vitamins such as vitamins C and E, folic acid, anthocyanins, glutathione (GSH), carotenoids (lycopene and carotene) and α-tocopherol, which are affected by this stress condition (Murshed *et al.*, 2013).

The aim of this study was to evaluate the effect of ozonated water with different doses of O_3 on the responses of (i) the enzymatic and nonenzymatic antioxidant systems of the fruit, (ii) physiological and biochemical properties and (iii) the microbial quality of a grape berry fruit salad.

Material and methods

Raw material

This study was performed with table grape cvs. Thompson Seedless (TS, white) and Black Seedless (BS, black) from Agrofruta Ltda. (Copiapó, Chile) at the Centro de Estudios Postcosecha (CEPOC) of the Facultad de Ciencias Agronómicas, Universidad de Chile (Santiago, Chile). At harvest, TS had 17.5% of total soluble solids (TSS) and titratable acidity (TA) of 5.9 g L^{-1} of tartaric acid, while BS had 20.8% of TSS and TA of 3.8 g L^{-1} of tartaric acid.

Minimal processing and treatments

Processing was performed in a conditioned handling room at 5 °C and began with selection of the raw material. The clusters were manually shelled, and the grapes were immediately immersed in cold tap water at 5 °C to reduce contact with O_2 and prevent oxidation of the peduncle insertion zone.

Subsequently, grapes were immersed for 4 min in cold ozonated water (5 °C) at different concentrations (2, 4, 6 or 8 mg L^{-1}). The ozonated water was obtained after generating O_3 in an ozonator (Magnum 25–160, Atlas, Canada) and later injecting the gas into cold water. Water O_3 concentrations were obtained by varying the oxygen flow rate and the working pressure of the equipment while final O_3 concentration was measured according to the method described by Bader & Hoigné (1981). The doses used were previously adjusted (data not shown).

As a control, a conventional treatment with 100 mg L^{-1} NaOCl solution (Clorox Chile SA, Santiago, Chile) was used. Disinfection treatments were performed in stainless steel containers of 50 L capacity using 10 L solution at 5 °C per kg of grapes, only once for each treatment into the tested concentration. Both grape cvs. (TS and BS) were placed together on stainless steel meshes for 2 min to drain excess water. From these meshes, three replicates of approximately 120 g of berries (mix: 60 g per cv.) were taken and packed into the same low-density polyethylene bags (40 μm thickness, 6000 mL O_2 m^{-2} d^{-1}). The bags (10 × 15 cm) were heat-sealed with a sealing machine (FR 400, HZPK, Zhejiang, China) and stored 21 days at 5 °C to simulate the storage and marketing period. After 1, 7, 14 and 21 days of processing, three bags of each treatment were randomly selected and removed from storage to be analysed.

Respiration rate

Respiration rate was determined in static system conditions by placing 120 g of grapes (mix: 60 g TS and 60 g BS) in a 500 mL glass container, which was tightly sealed and fitted with a silicone septum on its tap. After 2 h, gaseous headspace samples were taken through the silicone septum and injected into a gas chromatograph (Hewlett Packard 5890 Series II, Agilent Technologies, Wilmington, DE, USA) equipped with two packed columns arranged in series, the first an FT Porapak Q 80/100 mesh of 1.8 m × 1/4 in × 2 mm ID (Agilent, Wilmington, DE, USA) and the second a Molecular Sieve 13 × 80/100 mesh of 2 m × 1/8 in × 2 mm ID (Restek, Bellefonte, PA, USA), with a thermal conductivity detector (TCD). The working temperatures of the injector, oven and detector were 50, 50 and 200 °C, respectively. Helium (Indura, Santiago, Chile) was used as carrier gas at a working pressure of 300 kPa. As a calibration standard, a mixture of CO_2 (0.9 kPa), O_2 (18.2 kPa) and N_2 (81.5 kPa) provided by Indura was used. Respiration rate results were expressed as nmol CO_2 kg^{-1} s^{-1} for both cultivars mixed together, which is important information for choosing the proper plastic film to reach a recommended gas atmosphere in MAP.

Atmosphere composition

Measurements to identify changes in CO_2 and O_2 concentrations in each bag were performed using a portable gas analyzer (Checkpoint, PBI Dansensor, Ringsted, Denmark) previously calibrated by sampling atmospheric air (0% CO_2 and 21% O_2). Gas samples were taken through a silicon septum fixed outside the bag by an analyzer needle. The bags were changed on every sampling day. Values of O_2 and CO_2 registered by the analyzer were expressed as percentages.

Total polyphenol content (TPC)

Biochemical determinations were made on the skin of the berries and analysed separately for the black and white cultivars. The skin was used because previous studies showed that over 90% of TPC is located in it (D. Oyarzún, A.C. Silveira, unpublished data). After berry peeling, compound extraction from the skin was performed using the method reported by Singleton and Rossi (1965). Approximately 1 g of TS peel or 0.5 g of BS peel was weighed and homogenised separately with 9 mL of methanol for 1 min (T18 Ultra-Turrax, Shanghai, China).

Samples were stored in the dark for 24 h at 5 °C. Subsequently, the supernatant was filtered through four layers of cheesecloth and centrifuged for 20 min at 1050 ***g*** (Hermle Z 326 K, Hermle Labortechnink, Wehingen, Germany).

For the analysis, extraction aliquots of 19.2 µL were placed in Elisa plates (Jet Biofil, Shanghai, China) and 29 µL of 1 N Folin–Ciocalteu reagent was added. After 3 min in darkness, a mixture of 192 µL of NaOH (100 mM) and Na_2OH (317 mM) was added. Samples were incubated 1 h at room temperature and subsequently measured in triplicate in a microplate reader (Asys UVM-340, Biochrom, Cambridge, UK) at 750 nm. The results were expressed as g of gallic acid equivalents per kg of fresh weight (g kg^{-1}).

Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) was determined by the ferric reducing antioxidant power assay (FRAP) proposed by Benzie and Strain (1999) with some modifications and the DPPH antioxidant assay proposed by Brand-Williams *et al.* (1995) using the same extract as for TPC. For FRAP determination, Elisa plates (Jet Biofil) were used. In each well, aliquots of 6 µL of the extract and subsequently 198 µL of FRAP reagent were added. FRAP reagent was prepared with 300 mM acetate buffer (3.1 g $C_2H_3NaO_2{\cdot}3H_2O$ + 16 mL $C_2H_4O_2$ per L, pH 3.6), TPTZ (2, 4, 6-tripyridyl-s-triazine) solution (10 mM in 40 mM HCl) and ferric chloride ($FeCl_3{\cdot}6H_2O$) solution (20 mM in distilled water) at a proportion of 10:1:1 (v/v).

The mixtures were incubated 30 min in darkness at room temperature. Then, their absorbance was read on a microplate reader (Asys UVM-340) at 593 nm. The Elisa plate (Jet Biofil) was also used for DPPH (Sigma-Aldrich, St. Louis, MO, USA) determinations. In each well, 21 µL of extract and 94 µL of DPPH: methanol solution was added, and the plate was read at 515 nm. Three repetitions of each treatment were analysed, and results were expressed as g Trolox equivalent per kg of fresh weight (g kg^{-1}).

Oxidative enzymes

Protein determination

The protein concentration of the extracts was determined according to Bradford (1976) by measuring the optical density at 595 nm with bovine serum albumin as a standard.

Superoxide dismutase (SOD) determination

For enzyme extraction, 2.5 g of skin was mixed with 5 mL of extraction buffer Tris-HCl, 50 mM pH 7.5; 3 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 1 mM EDTA (Merck). Subsequently, the sample was homogenised for 1 min (T18 Ultra-Turrax) and then centrifuged for 20 min at 4 °C and 1050 ***g*** (Hermle Z 326 K, Hermle Labortechnink).

The supernatant was transferred to clean tubes protected from the light, and two identical Elisa plates (Jet Biofil) were prepared. Approximately 6 µL of enzyme extract was placed in each well, and subsequently, 351 µL of 50 mM phosphate buffer pH 7.8, 13 mM methionine (Merck), 75 mM nitro blue tetrazolium (NBT, Merck), 2 mM riboflavin (Merck) and 0.1 M EDTA (Merck) were added. One plate was used as a control and was stored for 15 min in darkness. As a blank, two plates with reaction buffer were used; one was placed in darkness and the other exposed to the light. To achieve NBT photoreduction, the light-exposed plates were placed 15 min under two 15 W lamps at a 30 cm distance. The absorbance of plates incubated in darkness and exposed to the light was measured at 560 and 593 nm, respectively, in a microplate reader (Asys UVM-340). The enzymatic activity was determined as the amount of enzyme that inhibited 50% of NBT photoreduction per kg of protein according to the methodology proposed by Dhindsa *et al.* (1981). Enzyme activity was expressed as activity units per mg of protein (U mg $protein^{-1}$).

Catalase (CAT) determination

One gram of peel, 0.2 g of polyvinyl pyrrolidone (Sigma-Aldrich) and 5 mL of phosphate potassium buffer 50 mM, pH 7.8 with 50 mM ethylenediamine tetraacetic acid (EDTA) (Merck), 0.1 mM L-cysteine 5 mM (Sigma-Aldrich), Triton X-100 at 3 mM (Calbiochem, USA) and 10 µL of phenyl sulfonyl fluoride per mL of extraction buffer were used. Subsequently, the mixture was homogenised for 5 min (T18 Ultra-Turrax), always maintaining the tubes on ice to prevent temperature increase.

Catalase determination was performed on 50 µL of the supernatant (extract) obtained after samples were centrifuged (20 000 *g*, 15 min and 4 °C). The extract was mixed with 1450 µL of potassium buffer, 50 mM pH 7 with 11 µL of H_2O_2 50 mM (Merck) in quartz cuvettes (Starna Cells, Inc., Atascadero, CA, USA). The absorbance was measured at 240 nm for 5 min in a spectrophotometer (UV-vis, T70, PG Instruments Limited, Leicestershire, UK). Three repetitions in triplicate of each treatment were analysed, and enzyme activity was expressed as units per mg of protein (U mg $protein^{-1}$).

Ascorbate peroxidase (APX) determination
Ascorbate peroxidase determination was performed using the same extract for CAT analysis (Starna Cells, Inc.) by mixing 505 μL of extract, 960 μL of phosphate potassium buffer 50 mM pH 7 with 0.1 mM EDTA (Merck), 0.5 mM ascorbic acid (Sigma-Aldrich) and 1.54 mM H_2O_2 (Merck). Samples were measured at 290 nm every 30 s in the spectrophotometer (UV-vis, T70, PG Instruments Limited) until the absorbance remained constant. Quartz cuvettes were used. Calculations of the U enzyme per mg of protein were made accounting for the decrease in absorbance until value stabilisation. Three repetitions, in triplicate of each treatment, were analysed. Enzyme activity was expressed as activity units per mg of protein (U mg protein^{-1}).

Microbiological analyses

For microbiological growth, 10 g of sample (peel and flesh) was homogenised for 2 min in 90 mL of sterile peptone water in a sterile bag (Easy Mix, AES Chemunex, Bruz, France) using a stomacher (Colorworth Stomacher 400, Seward, Worthing, UK). Serial dilutions were prepared according to the evolution of microbial counts. Total aerobic mesophilic and psychrotrophic counts were assessed on plate count agar (PCA, Merck, Darmstadt, Germany) and incubated for 2 days at 37 °C and 7 days at 5 °C, respectively.

Enterobacteriaceae enumeration was performed on violet red bile agar (VRBD, Merck) after a 48 h incubation at 37 °C, whereas moulds and yeast were assessed on acidified potato dextrose agar (Merck) with 110 mM lactic acid after 7 days of incubation at 25 °C. The results were expressed as log of colony-forming units per gram (log CFU g^{-1}). Analyses were performed on days 0, 1, 7, 14 and 21.

Statistical analysis

The experiment followed a completely randomised design. A bag containing 120 g of berries was used as a replication except for respiration rate measurements where a glass container was used instead of the bag. Each cultivar was independently analysed for biochemical parameters. Data were submitted to analysis of variance (ANOVA, $P \leq 0.05$). Two-way ANOVA ($P \leq 0.05$) was carried out considering treatments and storage time as factors. The results were reported as the mean ± standard error of three replicates. If statistically significant differences were identified among treatments, the means were separated by Tukey's test ($P \leq 0.05$). All statistical analyses were run in Infostat version 2012 (Universidad Nacional de Córdoba, Argentina).

Results and discussion

Respiration rate

Ozonated water treatments caused a significant initial increase in respiration rate, with two- to threefold increases compared to NaOCl-sanitised grapes (Fig. 1). This response was particularly noticeable after 5 days of storage at 5 °C where it reached its maximum. Then, O_3 immersion provoked additional stress beyond that generated by the processing.

The respiratory rate remained elevated and practically unchanged up to 13 days of storage. At day 17, the measured values decreased to almost half but continued to differ from NaOCl-sanitised grapes. At the end of storage, the respiration rate decreased without significant differences among treatments (ozonated water vs. control) with values between 6.19 and 10.13 nmol CO_2 kg^{-1} s^{-1}. NaOCl-sanitised grapes showed constant respiration throughout the storage with values ranging from 2.43 to 4.08 nmol CO_2 kg^{-1} s^{-1}.

The increased respiration of ozonated water-sanitised fruit could be because O_3 is a strong oxidant, which at the evaluated doses could cause some type of damage at the cellular level, which is expressed as increased respiratory activity. However, the response of plant tissues to the application of O_3 is highly variable. Many studies indicate that respiration is not affected by O_3. Arugula immersed in 10 mg L^{-1} ozonated water did not show any variation in respiration rate (Martínez-Sánchez *et al.*, 2008). In a similar way, the respiration rate of minimally processed Galia melon sanitised with 0.4 mg L^{-1} ozonated water for 3–5 min and stored 7 days at 5 °C was not noticeably

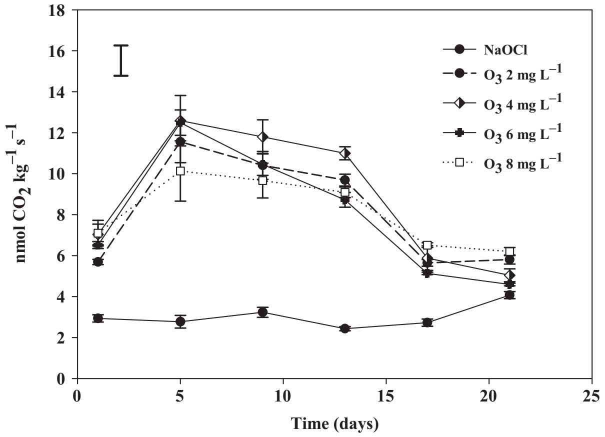

Figure 1 Respiration rate of minimally processed grape mix stored at 5 °C during 21 days (nmol CO2 kg^{-1} s^{-1}). Vertical bars indicate the standard error of the means (n = 3).

affected compared to NaOCl control treatment (Silveira *et al.*, 2010). It should be noted that the dose used on melon was almost ten times lower than the lower dose (2 mg L^{-1}) used in this case to sanitise grapes.

In addition, Restuccia *et al.* (2014) found a differential effect on the respiration rate of two artichoke cultivars immersed in ozonated water (2 mg L^{-1}). Artichoke Violet de Provence cv. sanitised with ozonated water showed an increased respiration rate compared to unwashed samples or those washed in tap water. However, artichoke Romanesco C3 cv. showed a slight reduction in respiration after O_3 treatment. In this sense, Zhang *et al.* (2005) observed that the respiration rate of minimally processed celery increased when O_3 concentration in water was increased; due to O_3 being a strong oxidant, the recommended doses could cause stress to vegetal tissues, which is expressed as increased respiratory activity.

The foregoing suggests that the effect of O_3 will depend on several factors such as the type of vegetal tissue, linked to the type of product and variety, the method of application (gas or water), the doses used, the exposure time and the water temperature among others.

Atmosphere composition

A decrease in O_2 and an increase in CO_2 concentrations were observed as a result of respiration and gas transfer through the polymeric matrix. However, in contrast to the behaviour observed for respiration, the CO_2 concentrations were similar for all treatments at the different storage times (data not shown). The values were 2.1–2.4% at the beginning of storage and 4.2–5.1% at the end of storage (day 21). In a similar way, O_2 concentrations showed no significant differences among treatments with values of 16.9–17.4% initially and 8.8–9.8% on day 21 (data not shown).

According to the O_2 (9–10%) and CO_2 (4–5%) concentrations reached, the characteristics of the film used to obtain modified atmosphere packaging (MAP), such as its permeability and especially its thickness, could mitigate the O_3 effect on respiration previously observed.

However, the gas concentrations inside the packaging allowed the grapes to maintain good appearance throughout the storage period. This could be due to the fact that MAP reduces the metabolic activity and maintains the overall quality of minimally processed products when CO_2 levels are higher than 1 kPa and O_2 levels are lower than 8% as reported in many studies (Waghmare *et al.*, 2013).

According to Del Nobile *et al.* (2009), minimally processed grapes stored at 5 °C in films of different material and thickness (20–105 μm) retained their quality for 35 days, even when 20 μm thickness was used, although the best results were observed with high barrier films (biodegradable monolayer and multilayer polyester-based co-extruded both of 100 μm).

On the other hand, Costa *et al.* (2011) preserved the quality of grapes for 70 days using an oriented polypropylene film of 80 μm thickness to achieve an O_2 concentration of 3%.

Total polyphenol content (TPC)

According to the results shown in Table 1, clear differences in TPC were found for both cvs. BS peel was richer in TPC and presented about seven times the contents measured in TS.

No interaction among treatment and storage time was found. In both cvs., TPC was significantly affected by ozonated water treatments. TS berries sanitised with NaOCl showed the lowest measured amount, while TS berries sanitised with 6 and 8 mg O_3 L^{-1} showed the highest content (Table 1). Additionally, a similar behaviour was observed in BS berries sanitised with ozonated water, where content ranged from 20.98 to 22.67 g kg^{-1} surpassing the amount measured on NaOCl-sanitised berries. Although a response proportional to the O_3 dose used was expected, nonsignificant differences among ozonated water treatments were observed.

Compared to NaOCl-sanitised berries, ozonated water treatments increased TPC by 23–58% and 18.5–28% in TS and BS, respectively. This could be due to the genetic differences in which greater postprocessing stress determined greater polyphenol synthesis in berries that naturally have a lower content compared to ones with high initial contents. Then, ozonated water treatments could be an interesting alternative to increase, to a certain extent, the amount of functional compounds in products with low content.

In general, aqueous or gaseous O_3 treatments increase polyphenol contents due to the stimulation of enzymes involved in the phenylpropanoid pathway

Table 1 Total polyphenols contents (g GAE kg^{-1}) of minimally processed Thompson Seedless and Black Seedless stored at 5 °C during 21 days

Treatment	Thompson Seedless	Black Seedless
NaOCl	*,†2.52 ± 0.09 C	17.71 ± 0.58 B
2 mg O_3 L^{-1}	3.11 ± 0.08 B	21.50 ± 0.52 A
4 mg O_3 L^{-1}	3.09 ± 0.09 B	20.98 ± 0.87 A
6 mg O_3 L^{-1}	3.74 ± 0.11 A	22.61 ± 0.71 A
8 mg O_3 L^{-1}	3.99 ± 0.09 A	22.67 ± 0.42 A

*Values are means (n = 3) ± standard error of the mean.

†Means followed by different letters, within the column, are statistically different according to Tukey's test at $P \leq 0.05$.

that rapidly increase their activity under O_3 exposure as defence mechanism operating in stress-affected cells (Dixon & Paiva, 1995). Additionally, Gill & Tuteja (2010) mentioned that an increased TPC was observed after O_3 exposure due to the stress generated by the oxidising properties of the gas itself and the radicals generated in its decomposition, which are stabilised by the phenolic compounds.

TPC increased after O_3 exposure was also reported on tomato (10 mg L^{-1} for 10 min) and artichokes immersed in 2 mg L^{-1} O_3, followed by storage at 4 °C under ozone-enriched atmosphere (0.1 mg O_3 L^{-1}) as reported by Rodoni *et al.*, 2010 and Restuccia *et al.*, 2014, respectively.

Total antioxidant activity (TAC)

TS showed a TAC (measured by the FRAP method) increased in berries-ozonated water sanitised as shown in Table 2. However, the response was not proportional to the dose as no differences between treatments were found. TAC was kept higher than the NaOCl-sanitised berries, reaching values up to 28% higher. Similar behaviour was registered on the values obtained by DPPH method, where grapes sanitised with 2–8 mg L^{-1} O_3 presented between 15% and 30% more TAC than NaOCl-sanitised grapes (Table 2). In contrast to FRAP method, in this case, differences between the higher and lower doses applied were found. In both methods, no interaction among treatment and storage time was found.

Additionally, TAC of BS showed a statistically significant differences among treatments and storage time. Using a DPPH method, a proportional increase according to the O_3 doses was observed. After 1 and 7 days of storage, berries treated with ozonated water at 6 and 8 mg L^{-1} O_3 increased twice their TAC compared to NaOCl-sanitised ones (Fig. 2a). After 14 and 21 days of storage, TAC was kept higher, reaching values up to 40% higher than the measured on NaOCl-sanitised berries. However, at 14 days, only the dose of 8 mg L^{-1} O_3 differed from 2 to 4 mg L^{-1} O_3, whereas at 21 days, no differences between doses were recorded.

Table 2 Total antioxidant capacity (g kg^{-1}) of minimally processed Black Seedless determined by DPPH and FRAP stored at 5 °C during 21 days

Treatment	FRAP assay	DPPH assay
NaOCl	*,†11.30 ± 0.34 B	40.60 ± 1.12 D
2 mg O_3 L^{-1}	13.46 ± 0.46 A	46.58 ± 1.45 C
4 mg O_3 L^{-1}	13.81 ± 0.3 A	47.01 ± 0.84 BC
6 mg O_3 L^{-1}	14.33 ± 0.53 A	51.81 ± 1.45 AB
8 mg O_3 L^{-1}	14.50 ± 0.55 A	52.74 ± 0.85 A

*Values are means (n = 3) ± standard error of the mean.

†Means followed by different letters, within the column, are statistically different according to Tukey's test at $P \leq 0.05$.

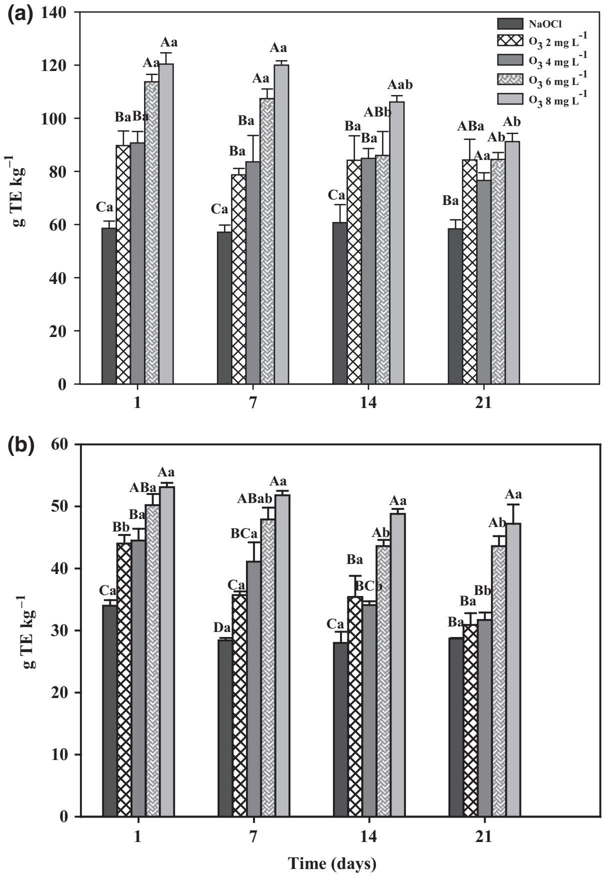

Figure 2 Total antioxidant capacity of minimally processed Thompson Seedless determined by DPPH (a) and FRAP (b) stored at 5 °C during 21 days. Vertical bars represent standard error of the means (n = 3). Means followed by different letters, uppercase for treatments and lowercase for time, are statistically different according to Tukey' test at $P \leq 0.05$.

Similar behaviour was registered on the values obtained by the FRAP method, where grapes sanitised with 6 and 8 mg L^{-1} O_3 increased their TAC values between 50% and 80% compared to NaOCl-sanitised berries, while the doses of 2 and 4 mg L^{-1} O_3 increased values for up to 30% (Fig. 2b).

The effect of ozonated water on TAC will depend on the type of compounds involved, whether they are positive or negative. The fact that TPCs were also affected by treatments with ozonated water would indicate that polyphenols are one of the main components responsible for the grape TAC.

The positive effect of O_3 on TAC, often linked to an increase in TPC, was reported in other studies in whole and minimally processed vegetables. In minimally processed Galia melon washed with ozonated water (0.4 mg L^{-1}) for 5 min, an increased TAC compared to control (NaOCl) was observed (Silveira *et al.*, 2010). In addition, Alothman *et al.* (2010) found an increase in TPC in minimally processed pineapple and banana after exposure to O_3 gas directly combined with an increase in the TAC. Therefore, the stress caused by a strong oxidising agent as O_3 activate the antioxidant system resulting in the improving of the antioxidant status of horticulture crops as mentioned by González-Aguilar *et al.* (2010).

Activity of oxidative stress enzymes

SOD activity

TS SOD activity did not show significant differences among treatments on 1 and 7 days (Fig. 3a). However, after 14 days, berries sanitised with 6 and 8 mg L^{-1} O_3 showed almost double the activity measured in the remaining treatments. These differences were not maintained; so after 21 days, no differences between treatments were observed.

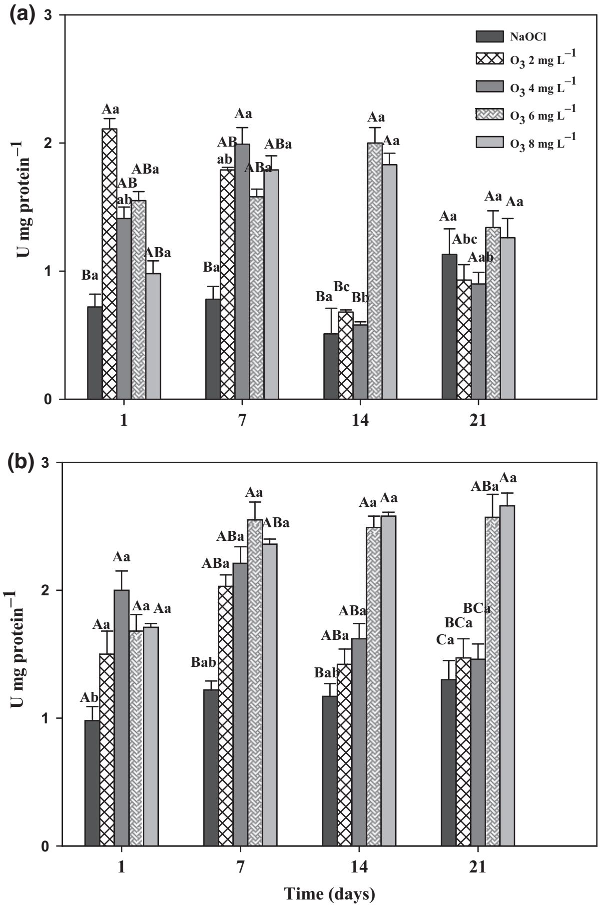

Figure 3 Superoxide dismutase (SOD) activity of minimally processed Thompson Seedless (a) and Black Seedless (b) stored at 5 °C during 21 days. Vertical bars represent standard error of the means ($n = 3$). Means followed by different letters, uppercase for treatments and lowercase for time, are statistically different according to Tukey' test at $P \leq 0.05$.

SOD activity was maintained with the advance of storage in berries sanitised with NaOCl and 6 and 8 mg L^{-1} O_3 whereas 2 and 4 mg L^{-1} O_3 sanitised berries showed a significant reduction from day 14.

At the beginning of the experiment, no differences among treatments were registered on BS berries sanitised with ozonated water and NaOCl (Fig. 3b). On day 7, 6 mg L^{-1} O_3 sanitised berries presented a significantly higher SOD activity compared to NaOCl.

Activity measured on day 14 and 21 on 6 and 8 mg L^{-1} O_3 sanitised berries was more than twice the measured on NaOCl. During 5 °C storage, SOD activity remained steadily in grapes immersed in ozonated water.

Comparing SOD activity in both cv., statistical differences were found (values not shown). The activity measured in BS, with an average value of 1.98 U mg protein^{-1}, exceeded the measure in TS with an average value of 1.35 U mg protein^{-1}.

CAT activity

In TS stored 7 and 14 days at 5 °C, O_3 produced an increase in CAT activity, achieving levels twofold higher than the registered on NaOCl-sanitised berries but without significant differences among O_3 doses (Fig. 4a). As observed in SOD, after 21 days, no differences between treatments were recorded. The enzymatic activity remained virtually constant along storage. However, berries sanitised with NaOCl showed an activity increasing after 21 days of storage.

BS showed the main CAT activity differences between the less oxidising treatment, NaOCl and the more oxidant one (8 mg L^{-1} of O_3) on 1, 7 and 21 days of storage. Contrary to expectations, no differences were found between O_3 doses (Fig. 4b). Related to the evolution of the activity of each treatment over time, a consistent pattern was not observed because in some treatments, the activity decreased along storage, while it increased in other treatments.

TS presented twice the activity measured in BS (values of 0.37 U mg protein^{-1} and 0.18 U mg protein^{-1}, respectively).

APX activity

No sanitizer treatments effect on APX activity was found both in TS as in BS (data not shown). TS presented an average of 58% more activity than BS (data not shown). In both cv., the activity increased with the course of storage. TS registered initial value of 0.5 U mg protein^{-1} and 0.73 U mg protein^{-1} after 21 days while BS showed an initial value of 0.3 U mg

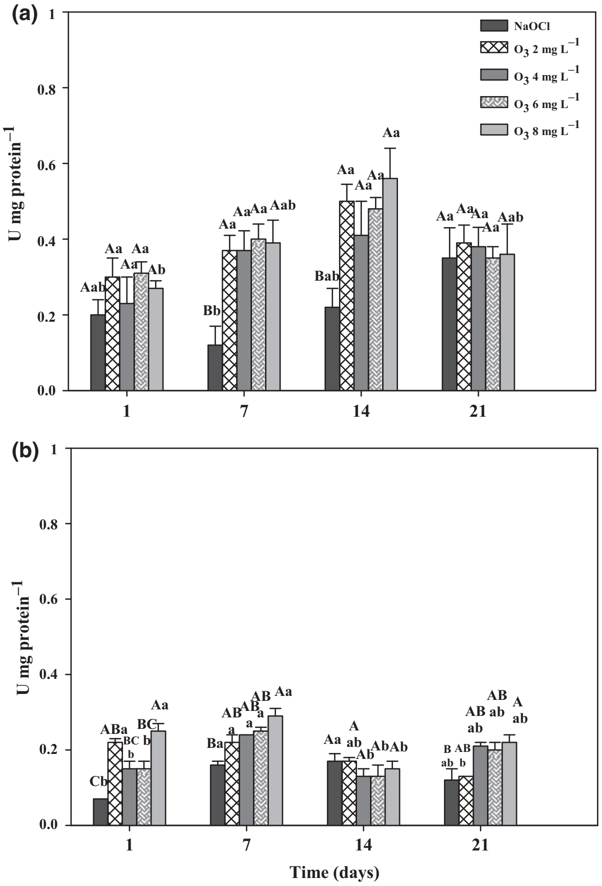

Figure 4 Catalase (CAT) activity of minimally processed Thompson Seedless (a) and Black Seedless (b) stored at 5 °C during 21 days. Vertical bars represent standard error of the means ($n = 3$). Means followed by different letters, uppercase for treatments and lowercase for time, are statistically different according to Tukey's test at $P \leq 0.05$.

protein^{-1} and 0.49 U mg protein^{-1} at the end of storage. This behaviour suggests that this enzyme was more affected by the senescence processes than by the stress caused by the sanitizers.

The activity of the different enzymes was not noticeably affected after 1 day at 5 °C. It was noted that main differences between treatments appear after 7 and 14 days of storage. This may be due to the fact that the stress response is not immediate as, after stress perception, an internal physical or chemical signal must be generated and transmitted to cell nucleus where changes in genes expression occurred to shovel the stress situation (Ben Rejeb *et al.*, 2014).

Although both, NaOCl and O_3, are oxidising agents, and therefore capable of generating stress, O_3 has a higher oxidising power which probably determined that the main difference between treatments was recorded between berries sanitised with NaOCl and ozonated water-sanitised ones. The increased enzyme activities observed in TS and BS may be due to a rise of ROS caused by the oxidising agents, which triggered a response to achieve detoxification.

Although it was expected that a higher dose would produce greater stress and therefore greater enzymatic activity, in most cases, this did not occur. This indicates that there would be a saturation of the response although additional studies could be undertaken to clarify this point.

The enzyme activities respond to an elevated ROS, potentially generated by the increased respiration rate that produces the superoxide radical ($O_2{\bullet}^-$), which was converted into hydrogen peroxide (H_2O_2) by SOD, the first enzyme to act against free radicals. This can be related to the finding of our study, which revealed that ozonated water treatments resulted in higher respiration rates compared to the NaOCl. SOD generates H_2O_2, potentially toxic specie for vegetal tissues, which is substrate for CAT, APX and POD that reduced of H_2O_2 levels below toxic concentrations (Mittler, 2002). Because of this, it was expected that an increase in SOD activity before CAT increase occurs, but this did not happen in all cases. In general, the increase in the activity of SOD and CAT occurred at the same time indicating that the mechanisms of enzymatic action are quite more complex than would be expected a priori.

The fact that BS variety, richer than TS in antioxidant compounds, would show higher CAT and APX activity would indicate a compensation of enzymatic and nonenzymatic antioxidant systems (Blokhina *et al.*, 2003; Xia *et al.*, 2016). The greater presence of antioxidant substances would compensate the lower activity of the enzymes.

Microbiological growth

The counts of the different microorganisms analysed were low and stable during the 21 days of the experiment. No significant differences were observed in psychrophilic and *Enterobacteriaceae* growth between treatments and analysis moments. After 21 days, at 5 °C, counts of these two microorganism groups were around 2 log CFU g^{-1} (data not shown). Additionally, mesophilic growth and mould and yeast counts presented values lower than 1 log CFU g^{-1} (data not shown).

The low counts recorded throughout storage at 5 °C were due to two factors, the low microbial load of the raw material along with the effectiveness of the sanitising methods used as previously reported (Silveira *et al.*, 2010; Bermúdez-Aguirre & Barbosa-Cánovas, 2013).

On the other hand, the physicochemical characteristics of the grapes, specially the low pH of fruit tissue which constitutes an unfavourable environment for bacteria growth, and the low impact of the unit operations performed (only shelled) also influenced in the low final load.

Conclusions

Immersion in ozonated water, especially when 6 and 8 mg L^{-1} were used, generates an additional stress to the vegetal grape tissue that favoured the activation of enzymatic and nonenzymatic antioxidant stress systems to achieve detoxification.

Grapes sanitised with ozonated water showed high total polyphenol contents and antioxidant capacity with similar microbial counts compared to NaOCl used by the industry. Therefore, ozonated water in doses of 6 and 8 mg L^{-1} could be an alternative to maintain low microbial counts and reach high functional contents that are beneficial for the consumer.

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