Research Article

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Ozone gas as a storage treatment to control *Gnomoniopsis castanea*, preserving chestnut quality

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Abstract

BACKGROUND: Chestnuts are gluten-free, low-fat, cholesterol-free products. Postharvest decay reduces chestnut shelf life and can cause severe economic losses. In this study we investigated the effect of ozone (O_3) gaseous treatment on chestnut rot caused by *Gnomoniopsis castanea* and the quality parameters of chestnuts.

RESULTS: The results showed that ozone treatment (150 ppb during the day, and 300 ppb during the night) reduced the decay of chestnuts and had a fungistatic effect on isolates of *G. castanea*. The exposure of chestnuts to ozone did not alter weight losses, sugar content and titratable acidity. The concentration of total phenolics decreased during the storage period, both for treated and untreated nuts. However, after 150 days of treatment the polyphenol content of the chestnuts exposed to ozone was significantly higher than in control nuts.

CONCLUSIONS: Our results suggested that ozone is an appropriate and economical tool to maximize the quality of chestnut shelf life, enabling it to be stored for long periods.

Keywords: ozone as gas; *Gnomoniopsis castanea*; *Castanea sativa*; cold storage; quality parameters

INTRODUCTION

Sweet chestnut (*Castanea sativa* Mill.) is a native European species appreciated from ancient times for its many uses. It was treasured for its versatile and useful wood and nut production during Roman times, when the Romans started to use it as fruit plant and to cultivate it across Europe. This increased during the Christian age. In a short period, it became one of the main sources of energy for human beings. Nuts are rich in starch (about 50%), sucrose, glucose, and fructose (about 10–22%, up to 1.2%, and up to 0.8%, respectively, on a dry matter basis), and raffinose.[1–3] Due to their chemical characteristics nuts consumption offers positive health benefits: reduction of plasma lipid levels – cholesterols, very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) – increase of the lipids peroxidability and vitamin E production.[4]

Chestnut cultivation still represents an important source of income, notably for disadvantaged areas (remote, mountainous, and sparsely populated areas). Currently, Europe accounts for 17% of world chestnut production, with Italy, Turkey and Portugal as the main producer countries (30%, 29%, and 15% of the European chestnut production, respectively). Because of its importance, a lot more attention should be given to adress the development of management strategies and the chestnut trade.

Chestnuts, and in particular the 'Marron' variety, are consumed mainly as fresh or roasted products. Unfortunately, the large moisture (about 50% of water) and sugar content, enzyme activity, and pericarp characteristics result in a very limited shelf life for these fruits. Moreover, pests and pathogens infecting chestnuts before harvesting, continue to affect them during storage.[5] Insect damage is usually due to infestations of *Cydia* spp. and *Curculio elephas* in pre-harvesting. Several fungi also colonize the fruits at various stages during their development and can cause nut rot of chestnut in preharvest and / or postharvest conditions.[6–8] In recent years, an increase in the incidence of rotten chestnuts, mainly caused by *Gnomoniopsis castanea* Tamietti, has been observed in Europe.[7,9,10] In a few years, brown fruit rot has become the most important disease in chestnut fruits, sometimes compromising more than 50% of production.[11] The pathogen, *G. castanea*, causes the kernel of affected nuts to turn chalky, white, and spongelike, resulting in browning of the fruit and an alteration in the flavor,[7] with consequent important economic losses.

Thus, the development of effective postharvest treatments, storage conditions, and packaging systems is needed to minimize spoilage, maintain the quality of chestnuts, and extend their shelf life.

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The most widely used chemical treatments pose serious threats for human health and environment and were banned for use in the European Union under the Montreal Protocol. In a scenario of sustainable chestnut chain production, it is therefore crucial to develop alternative strategies. Several different postharvest environmentally friendly methods have been applied to preserve the nutritional and sensory properties of the fruits and vegetables, to keep the products fresh and to protect them against physiological and biological losses during postharvest periods.[12,13] Specifically, for the storage of chestnuts, researchers tested, among other methods, the use of radio frequencies, controlled atmospheres[10,14–17] and gamma radiation.[18]

Currently, water curing ('curatura') is the commonly used postharvest method. It is based on soaking fruits in cold water (hydrotherapy) for about 1 week or hot water (50 °C; warm bath) for about 45 min, and cooling them in water at 15–18 °C, again for 1 week.[11,15,19] Water curing treatment permits insect-infected nuts to be separated. It can also have the disadvantage of lowering the sweetness and aroma of chestnuts, especially in the case of the cold bath[6] and can be very work intensive and more expensive in the case of the warm bath.

Recently, much more attention has been focused on the use of ozone (O_3) as a tool for limiting losses in product quality.[20] Ozone is a naturally occurring gas in the atmosphere, generated by the passage of oxygen (O_2) gas through a high-voltage electrical discharge or by ultraviolet light irradiation.[21] It can be applied either as a gas or diluted in water. The application of O_3 as a direct food additive for the treatment, storage, and processing of foods has been approved by the Food and Drug Administration.[22]

Several studies have focused on the effect of O_3 treatments on quality-related attributes and microbial growth on fruits and vegetables.[23–27] In general, nutritional and quality parameters of fruit and vegetables (such as respiration rate, ethylene emission, weight loss, color, glucose, fructose, sucrose, organic acid, and antioxidant content) were maintained or increased following O_3 exposure.[28–30] Nevertheless, the high oxidation power of ozone could also lead to undesirable changes in the fruit quality, due to the high moisture content, enzymes, and phenolic compounds in the fruit.[31,32] Ozone has been reported to reduce or delay the decay of fruits and vegetables.[33] However, its efficacy is variable, and depends on the protocol used and the host and pathogen studied.[33,34] Unlike many other fruits, little information is available on the efficacy of sanitation treatments of chestnuts using ozone as a gas. Lee *et al.* (2016)[35] and Donis-González *et al.* (2016)[36] reported that treatments of *Castanea crenata* and 'Colossal' cultivar with ozone solution may play an important role in lowering the incidence of decayed kernels. As far as the authors are aware, the effectiveness of treatments with ozone as gas on *C. sativa* is unknown. An experiment was therefore performed to evaluate the efficacy of treatment with ozone as a gas to extend the storage of chestnuts and specifically to preserve all qualitative characteristics of the fruit and to reduce or eradicate the decay caused by *G. castanea*, the major causal agent of nut rot in Europe.

MATERIAL AND METHODS

Samples

Nuts (200 kg) were randomly and manually collected in a commercial chestnut field located in Monti Cimini, Viterbo, Italy (800 m a.s.l, 42° 21′ 29.52″ N 12° 10′ 39.72″ E) at harvest time, and immediately processed. Trials were arranged in a completely randomized block design with five replicates, each replicate composed of three plants. Once in the laboratory, a subsample with no visual defects and a uniformity of weight and shape, was sterilized (60 s in 75% ethanol (Sigma Aldrich, Milan, Italy), 3 min in 3% NaClO, and 30 s in 75% ethanol) and rinsed in sterile distilled water for the experimental tests. These treatments were conducted to avoid the growth of molds on the pericarp of nuts.

Treatments and storage

Nuts were randomly arranged in two groups: (a) control chestnuts (CK) kept in air, and (b) chestnuts maintained under a continuous flow of ozone in air (150 ppb during the day, and 300 ppb during the night) (O_3). For both groups, storage temperature was kept at 2.0 ± 0.5 °C; while relative humidity (RH) was settled at $95 \pm 2.0\%$, as suggested by previous studies.[6] For each group, 50 kg of chestnuts were equally distributed in 10 plastic perforated trays (40 x 60 x 10 cm; Agricola Imballaggi S.r.l., Sa, Italy). During the experiment, chestnuts were turned over every 3 days. Ozone was generated using a commercial ozone generator (model C32-AG, Industrie De Nora Spa, Milan, Italy) equipped with an oxygen concentrator (nominal production capacity of 32 g O_3 h^{-1}). The gas was delivered directly in the cold room and continuously measured using an UV-photometric ozone analyzer (BMT 146 Messtechnik Gmbh, DE). Temperature and RH in each of the two treatment rooms were monitored using a data logger (mod. 175 H1, Testo s.p.a, Mi, Italy).

Quality analysis

Quality analysis was performed every 30 days over 5 months of treatment, both for CK and O_3 sets (October 2016 – February 2017). The effects of the ozone treatment were investigated by physical, chemical, and biological methods, measuring weight loss (WL), solid soluble content (SSC), titratable acidity (TA), total polyphenol content, and the incidence of decay caused by *G. castanea*.

Weight loss

Before storage and at the end of the experiment, 200 chestnuts were removed for treatment and replicates to determine the nut weight. Weight loss was monitored using a technical balance (Adam Equipment Co. Ltd., Milton Keynes, UK). The percentage of weight loss (WL) was determined according to Eqn 1:

$$WL = (W^0 - W^t/W^0) * 100. \quad (1)$$

where W_0 is the initial sample mass and W_t is the sample mass at time t.

Chemical analysis

At each sampling time, 1 kg of chestnuts was used for chemical analysis. Five g of fruit pulp randomly collected was added to 10 mL of distilled water and homogenized with an Ultra-Turrax at 3000 rpm for 30 s.[19] Several rates (2 mL each) of the homogenate were centrifuged using the Eppendorf tubes. The surnatant was used for the following analysis: (a) SSC was measured using a digital refractometer (ATAGO Co., Japan), a few drops of the supernatant were used to read the refractometer (°Brix); (b) TA was determined with 0.1 mol L^{-1} NaOH and results were expressed as mg citric acid per 100 g fresh weight (FW); (c) TP of fruit pulp was determined as described by Botondi *et al.* (2009)[19] using a Lambda 25 UV/VIS spectrophotometer (PerkinElmer Ltd., UK) at 700 nm. The total polyphenols were detected on the basis of a gallic acid standard curve (mg GAE per 100 g FW).

Incidence of decay

At the beginning of the experiments (T0), 200 nuts, randomly chosen, were cut in half and visually assessed for the presence or absence of fruit rot. The visual assessment was repeated after 5 months of storage at 2 °C, on 200 CK and 200 O_3 treated chestnuts. Each fruit was classified into one of the following two categories: healthy or mold damaged. The incidence of decay was estimated as the percentage of chestnuts showing brown rot symptoms.

Isolation of G. castanea *and* in vitro *inhibition tests*

The endosperm of each chestnut, visually assessed for the presence of the decay, was cut into small fragments (5 × 5 mm). To confirm the presence of *G. castanea,* five fragments from each nut, symptomatic or asymptomatic, were plated onto Petri dishes containing potato dextrose agar (PDA, Oxoid, UK, 39 g/L^{-1}) amended with streptomycin sulfate (0.06 g/L^{-1}). After 1 week at 22 ± 2 °C, plates were observed. From a representative number of colonies resembling *G. castanea* (10%) monohyphal isolates were obtained and identified on the base of morphological and molecular traits.[37,38] Isolates Pini a and Pini d, previously identified as *G. castanea,*[37] were used as references. Isolates recorded as *G. castanea* were counted, transferred on PDA plates, and maintained at 24 °C.

Mycelial plugs (8 mm) from actively growing colonies of the isolates Gc1 and Gc2, obtained in this study and chosen as representative, were placed on PDA plates, incubated at 4 °C in the absence of ozone (GC), and treated with ozone at different times of exposition (2, 4, 8, 16, 24 and 72 h) (GT). To evaluate the fungicide/fungistatic effect of ozone treatments, after each experiment, plates were transferred at 20 °C. Fungal growth was monitored daily for 24 days. The *X* and *Y* axes of the mycelial growth were measured in millimeters. An average of the *X* and *Y* axes was used to obtain the average diameter of mycelia growth.

Statistical analysis

Data were analyzed using one-factor analysis of variance (ANOVA). Significance between means was determined using the Tukey post hoc multiple comparisons of means test at the 95% family-wise confidence level ($P = 0.05$). All statistical analyses were performed using Sigma Plot 13 (Trial Version, Systat Software, San Jose, CA, USA).

RESULTS AND DISCUSSION

As far as the authors are aware, this is the first study about the effects of O_3 utilized for gas treatment of chestnuts during postharvest storage. The data obtained during the present study showed that the use of O_3 during postharvest storage can affect the quality of chestnuts.

Weight loss

The treatment with ozone had no effect on chestnut weight loss during storage. Weight loss of nuts increased linearly by about 2.2 ± 0.5% per month, both in treatments with and without ozone ($P > 0.05$) (Table 1). These findings agree with results of Donis González *et al.* (2010)[39] showing that water treatments with ozone do not reduce chestnuts weight loss during storage. For strawberries and kiwi exposed to ozone at 0.08 g L^{-1} and 2.8–9.3 g L^{-1}, respectively[40,41] the weight was reduced. The different behavior among fruits should be due to the fact that water loss from fruits and vegetables occurs mainly through the cuticle.[42] Thus, its thickness and composition could lead to reduced membrane damage and skin-surface cracking and consequently reduced water loss.[43,44] The rapidity with which water is lost is also related to the pressure gradient between the fruit and surrounding atmosphere.[40]

Chemical analysis

During storage, fruits are subjected to many unfavorable processes that cause sugar metabolization and titratable acidity changes. The evaluation of TA is a suitable way to provide evidence of the evolution of the organic acid content during fruits storage. Its increasing level may indicate improper storage conditions.[45] In our experiment, the ozone treatment did not produce any undesirable effects on sugar and acidity content (Table 1). Solid soluble content increased from a concentration of 8.4 ± 0.8 °Brix at the beginning of the experiment up to 12.4 ± 0.8 °Brix and 13.0 ± 0.9 for CK and O_3 samples, respectively, after 5 months (Table 1). Chenlo *et al.* (2010) also observed that, in chestnuts maintained at 3 °C in a

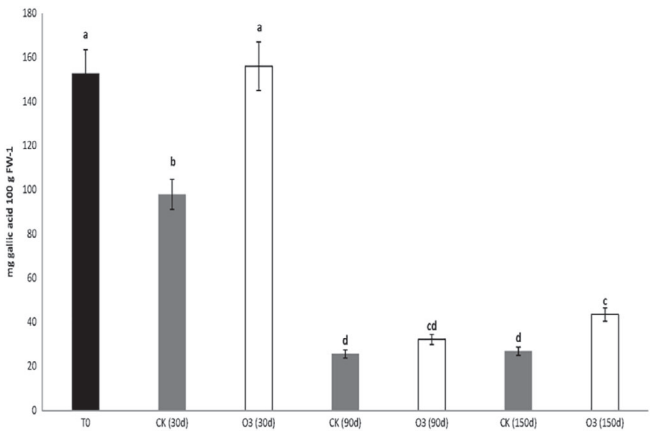

Figure 1. Polyphenol content (mg GAE per 100 g FW) of CK and O_3 chestnut fruits during cold storage at 2 °C. Values are the mean three replicate samples ± standard error. The same letters indicate a significant difference between the polyphenol content at $P \leq 0.05$.

Table 1. Changes in weight loss (WL) titratable acidity (TA) and solid soluble content (SSC) of chestnuts untreated (CK) and treated with ozone (O_3) for 150 days

		30 Days		60 Days		120 Days		150 Days	
	Day 0	CK	O_3	CK	O_3	CK	O_3	CK	O_3
WL	-	$2.2^{a} \pm 0.6$	$2.1^{a} \pm 0.4$	$1.9^{a} \pm 0.5$	$2.0^{a} \pm 0.4$	$2.1^{a} \pm 0.4$	$2.2^{a} \pm 0.5$	$2.5^{a} \pm 0.6$	$2.6^{a} \pm 0.6$
TA	$31.4^{a} \pm 6.2$	$29.2^{a} \pm 4.8$	$29.3^{a} \pm 5.8$	$28.8^{a} \pm 4.5$	$28.6^{a} \pm 3.4$	$28.6^{a} \pm 5.8$	$28.1^{a} \pm 4.8$	$26.5^{a} \pm 3.9$	$27.1^{a} \pm 5.0$
SSC	$8.4^{b} \pm 0.8$	$9.3^{ab} \pm 1.3$	$9.6^{ab} \pm 1.1$	$10.4^{ab} \pm 1.2$	$10.5^{ab} \pm 1.0$	$12.6^{a} \pm 0.9$	$11.9^{a} \pm 1.0$	$12.4^{a} \pm 0.8$	$12.6^{a} \pm 0.9$

Data are presented as the mean ± error standard. The same letter in each line indicates no significant differences among the data at $P \leq 0.05$.

normal atmosphere, sucrose content reached values 60% higher than the initial values, after 30 days of storage.[46] This characteristic could be particularly appreciated by consumers.

Titratable acidity remained constant, ranging from 31.4 ± 6.2 (T0) to 26.5 ± 3.9 (CK after 150 days) (ANOVA; $P > 0.05$) (Table 1). The results corroborate data shown by Botondi *et al.*, (2018).[27] An opposite tendency has been reported for ozonated papayas by Ali *et al.* (2014),[47] where a drop in the organic acid content after treatment with O_3 at 1.5 and 5 ppm was recorded.

In our study, the most notable effect of ozone on chemical quality of chestnuts was the changes in total polyphenols, both in CK and O_3-treated chestnuts. After 1 month a decrease in the total polyphenols was observed only in the CK samples (Fig. 1), as already reported for nuts of the variety *C. henryi* by Xu (2005).[48] The rapid and drastic decrease in polyphenol content during air storage might be the result of polyphenol oxidation because these antioxidants are not strongly bound but are free in the tissue due to water diffusion. Thus, they are more sensitive to the effect of environmental air.

The minimum total phenols values (27.5 ± 2.8 mg GAE per 100 g FW) were reached after 90 days. Ozone had no effect on the polyphenol content within 30 days of storage. However, the TP concentration significantly dropped by about 24% after 90 days of storage (ANOVA; $P < 0.05$). Although treatment at 2 °C caused a rapid decrease in the polyphenols, at the end of the experiment the polyphenol content of O_3 was significantly higher (40%) than that of CK samples. These results could be due to the fact that continuous gaseous ozone treatment can activate polyphenol synthesis as a defense response.[48] It is now well known that the metabolism of polyphenols and the polyphenol content may be affected by the concentration of ozone.[25] The variation in the polyphenol content at different dosages of ozone exposure could be explained either by the increased activity of phenylalanine ammonia lyase, or by a decrease in polyphenol oxidase and peroxidase activities.[25]

Phytopathological analysis

In this study, the incidence of chestnuts decay at harvest was about 19% (38 nuts of 200 analyzed), a value that is comparable with those found in the Monti Cimini area over the last few years. As expected, after a long period of storage at 2 °C, the number of affected nuts increased, reaching a total of 25% and 87% in storage with ozone and without treatment, respectively. The ozone treatment maintained the nuts' loss around values close to those registered at harvesting time (ANOVA; $P > 0.05$). However, fruits treated with ozone showed a significant lower incidence of decay compared to the CK group (chi-square = 6.34; $P = 0.001$). In a previous study, Lee *et al.* (2016)[34] showed that washing treatments with ozone reduced the frequency of decayed chestnuts and bacteria and fungi colonization of the pericarp of chestnuts (*C. crenata* 'Tsukuba'). However, according to Donis González *et al.* (2009)[39] ozone treatments are less effective for reducing mold frequency than other chemical sanitizers, such as hydrogen peroxide, peracetic acid, and trifloxystrobin. Although the action of ozone is on the outer surface, it is evident that it can penetrate into the nuts and impact the microbioma present in the endosperm. *Gnomoniopsis castanea* continued growing during the storage period of 150 days. It was isolated from about 40% of the nuts at the beginning of the experiment and the frequency of isolation increased significantly to 75% after 5 months of treatment, both for O_3 and CK groups (chi-square, $P > 0.05$). Colonies were always isolated both from asymptomatic and symptomatic nuts. The fungal growth of *G. castanea* not exposed to ozone treatment and maintained at 2 °C was characterized by an initial lag phase (adaptation period) of about 10 days, followed by a linear growth (Fig. 2a). In an *in vitro* test, ozone treatment inhibited mycelia growth at each application time. In fact, regardlethess of exposure time to ozone, colonies once transferred at 20 °C started growing after 24 days. Thus, at this concentration, ozone had a fungistatic and not fungicidal effect. This action was more evident on isolates treated with ozone for more than 4 h (Fig. 2b).

This study focused on *G. castanea*, so inferences cannot be drawn about the effectiveness of ozone on other pests and pathogens present in the nuts. In previous studies, it is reported that the microbial population of the pericarp and kernel of chestnuts are reduced in fruits exposed to O_3. It is worth noticing that unlike this research above cited studies relate to different chestnut varieties, *C. crenata* and the 'Colossal' cultivar collected in Michigan and South Korea and exposed to ozone solution.

CONCLUSION

Chestnuts are seasonal fruits, consumed mainly as a fresh or roasted fruit. The possibility of increasing their shelf life would be of the international huge benefit to the producers, enhancing local commerce and trade. The preliminary findings of this study illustrate the validity of using gaseous ozone as a tool to maintain

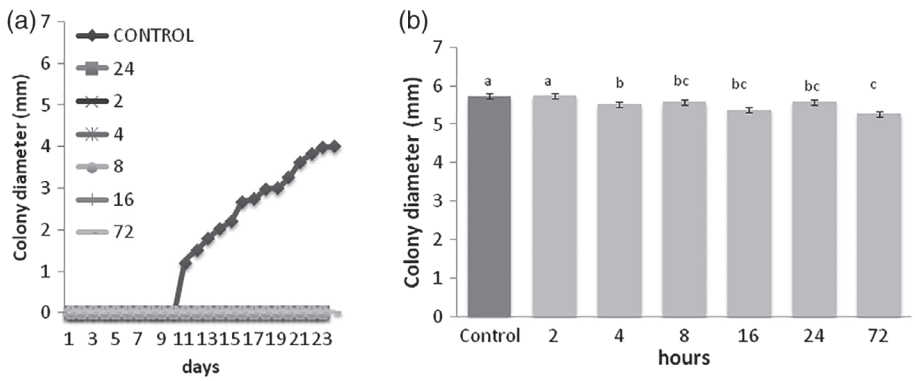

Figure 2. Growth of *G. castanea* colonies, not treated (dark gray line) and treated at different exposure time with ozone and maintained at 2°C (a). Error bars represent the standard error of the mean. Numbers in the legend indicate hours of exposure to ozone. Growth of *G. castanea* colonies transferred at 20 °C after treatments with ozone for 0 (control), 2, 4, 8, 16, 24, and 72 h. Lower case letters indicate a significant difference between the colony's growth at $P \leq 0.05$.

the high quality of chestnuts over a long storage period. Unfortunately, little is known about the effects of O_3 treatments on chestnuts or similar products. Thus, data obtained from previous studies on other fruits or vegetables can provide only approximate information. Further studies are ongoing to optimize treatments of chestnuts with ozone, alone or together with other products, which could provide an effective, cheap and easily applicable solution for chestnut farmers, compatible with customer demand, due to the absence of chemical residue.

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