Preliminary studies on microbial management efficiency of ozonated water on Italian ready-to-eat table grape variety

Marco Vendemia[1], Gabriele Caponio[1], Francesca Ferrulli[1], Leonardo Scarano[1], Rocco Perniola[1], Antonio Coletta[1], and Antonio Domenico Marsico[1]

[1]Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria - Centro di Ricerca Viticoltura ed Enologia (CREA-VE), Via Casamassima 148, 70010 Turi, Italy

Abstract. Ozonated water is an alternative means of post-harvest fruit and vegetable management that keeps gaining interest for its applications. The aim of this study was to evaluate the effects of ozonated water at different concentrations (12 mg L^{-1} and 8 mg L^{-1}) on ready-to-eat Italian 'Regal seedless' grapes, to assess the ozone effect on grey mould and berry microbiome (non-*Saccharomyces* yeasts, total bacteria, and total fungi). An ozone generator capable of producing ozone concentration ranging from 18 to 65 Nm^3 was used to obtain the different ozone concentration levels in water where berries were immersed. After 26 days of cold storage, grey mould incidence was assessed as percentage ratio between the number of affected berries and the total number of berries. Berries dipped into ozonated water at the higher ozone concentration of 12 mg L^{-1} showed a 61% average reduction of the overall disease incidence compared to the control. Moreover, the microbiome of berries treated with 12 mg L^{-1} ozone concentration showed significant reduction of fungal and yeast populations, while not showing any significant difference for the bacterial population, compared to the control.

1 Introduction

Grapevine is one of the most widespread crops in the world, with an attested production of fresh table grapes around 30.1 mt (million tons), with Italy being one of the top 10 producers [1].

Table grape hosts a complex microbiome on its surface, including yeasts, bacteria, and fungi, some of which have been proving themselves useful in terms of technological applications [2-6]. Due to the non-climacteric nature and delicate structure of grape berries, however, poor management of post-harvest and cold storage treatments could also lead to alterations such as stem browning, berry drop and microbial rots [7-11], undermining their chemico-physical qualities and the shelf-life. With particular emphasis in humid, subtropical areas, the most dangerous microbial disease is the grey mould of grapevine caused by the fungus *Botrytis cinerea* Pers.: Fr (*Botryotinia fuckeliana* (de Bary) Whetzel) [12], because of its capability to grow and spread below -0.5 °C when already present [13,14].

At the time, sulphur dioxide (SO_2) in the form of sodium metabisulphite ($Na_2S_2O_5$) generating pads became the most adopted solution to control the disease and the epiphytic microbiome. Effective antimicrobial and antiseptic properties of the molecule are associated to affordability, ease of use and less health risks compared to traditional fungicides [7,15-17]. However, factors such as the increase in the concerns around consequences towards human health and the environment, and the spread of the 'organic' approach to viticulture, have hasted the development of alternative, more sustainable strategies to ensure a better post-harvest management of grape berries rather than sulphur dioxide. Different means of control have been proposed and tested during the years, spacing from biological [18], physical [18,19] and chemical [20] solutions, among which ozone treatments have been received more and more attention for its eco-friendly advantages and sanitizing properties on different crops [21-26].

Ozone (O_3) is a GRAS-type decontaminating gas capable of an interesting antimicrobial activity due to its strong oxidizing potential on different cellular constituents [27]. Its most competitive technological advantage, however, is a rapid decomposition that results in a lack of harmful residues on the treated fruit surfaces [28]: the molecule spontaneously decays back to oxygen (O_2) because of its high chemical instability, either when in gaseous phase (half-life at 20 °C: 3 days) or in aqueous phase (half-life: 20 min) [29,30].

The aim of the study was then to evaluate the sanitizing effect and bioprotective qualities of ozonated

water at different concentrations on the ready-to-eat Italian table grape variety 'Regal seedless', producing preliminary assessment data about the control of the grey mould of grapevine, effects on the berry local microbiome in semi-commercial conditions, and the consequences on table grape shelf-life from the first steps of the production chain.

2 Materials and methods

2.1 Grapevines and ozone treatments

The experiments were conducted on 'Regal seedless' grape bunches harvested during the 2022 season from a commercial vineyard located in Casamassima (Apulia region, Italy). Berries from visible healthy bunches were collected, deprived from their pedicel, and placed in perforated plastic clamshell boxes reaching a volume of 100 g each.

Ozonated water at the desired concentrations of 12 mg L^{-1} and 8 mg L^{-1} was obtained on site (room temperature: 17 °C) with an ozone generator capable of producing ozone concentrations ranging from 18 Nm^3 to 65 Nm^3. The resulting ozonated water was used to fill a 70 L plastic washing tank via a circulation pump and constantly monitored through an ozone analyser (Fig. 1).

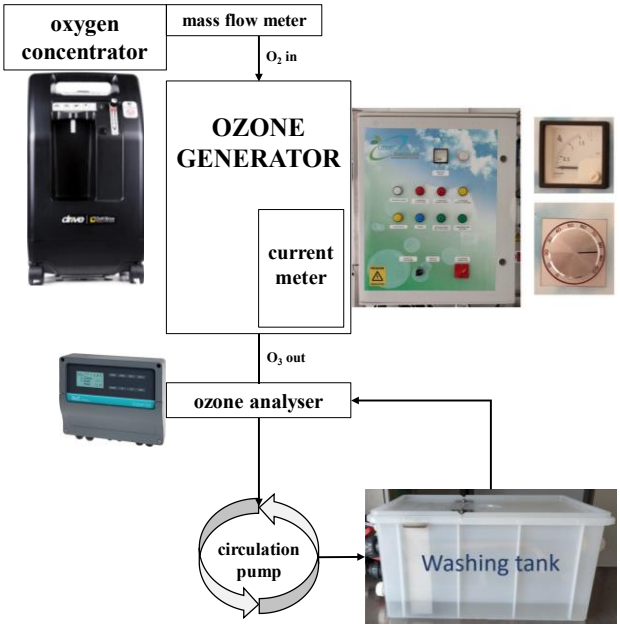

Figure 1. Ozonated water production workflow.

The effect of the ozonated water at the two concentrations was tested on 8 replicates. The berries contained in the perforated boxes were dipped into each ozone concentration level for 5 minutes (T1: 12 mg L^{-1} × 5'; T2: 8 mg L^{-1} × 5'). For this experiment, another batch of 8 boxes was dipped in tap water for 5 minutes to be used as control (C: 0 mg L^{-1} × 5') (Fig. 2). All the boxes were then thermally sealed and cold stored at 1 °C and a 95% relative humidity (RH) for 26 days.

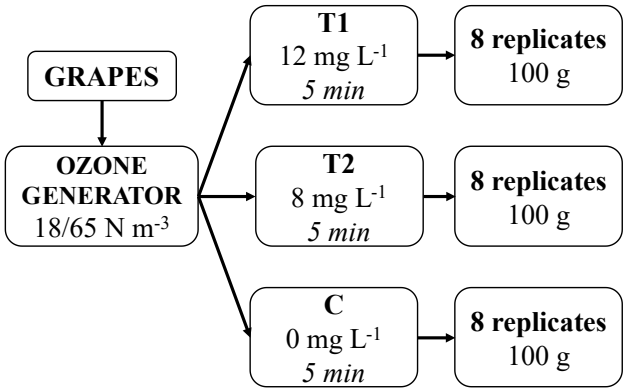

Figure 2. Experimental design and treatment codes for the different theses.

2.1 Evaluation of the disease incidence of *B. cinerea*

After the cold storage period of 26 days, a visual evaluation of the presence of the grey mould causal agent *B. cinerea* on the berries surface was performed. The average grey mould incidence was calculated as percentage ratio between the number of infected berries and the total number of berries:

$$DI = n \text{ infected berries} / n \text{ total berries} \quad (1)$$

The DI value was then adopted to calculate the efficiency of each treatment, expressed with the formula:

$$E = DI_{TI} - DI_C / DI_C \quad (2)$$

2.2 Biological assessment of the berry microbiome

10 g of berries from each thesis showing no sign of exterior damage was placed in a large beaker containing 200 mL of sterile Ringer solution (NaCl 2.25 g, KCl 0.01 g, $CaCl_2$ 0.12 g, $NaHCO_3$ 0.05 g, TWEEN 20 four drops) under aseptically conditions. The beaker was put under agitation for 30', to allow the microorganisms separation from the berry skin. The resultant microbial suspension (mother solution) was serially 1-to-10 diluted in sterile plastic vials, where 1 mL of the suspension was eluted in 9 mL of sterile Ringer solution three times up to a 10^{-3} CFU mL^{-1} concentration. Appropriate volumes of the diluted microbial suspensions were subsequently plated onto selective solid growth media to evaluate the nature of the epiphytic microbiome: 200 µL on Wallerstein Laboratory (WL) Nutrient Medium (VWR Chemicals, Leuven, Belgium) for yeast populations; 100 µL on Nutrient Broth (NB) (NaCl 5 g, meat peptone 5 g, yeast extract 2 g, agar powder 16 g) for bacteria; 500 µL on Trichoderma Selective Medium (TSM) (KCl 0.151 g, K_2HPO_4 0.9 g, $MgSO_4$ 0.2 g, NH_4NO_3 3 g, glucose 3 g, Bengal rose 0.08 g, agar 20 g, ampicillin 790 µL, streptomycin 1 mL) for fungi. Plates were incubated at

25°C for 3 days, to allow the colonies to reach the diameter of 0.25 ± 0.05 mm. After 3 days, the colonies were counted and the number of Colony Forming Units (CFU) mL^{-1} was estimated.

2.3 Statistical analysis

Collected data were used to perform a one- way ANOVA test, followed by the post- hoc Tukey's test ($p < 0.05$). Data were analysed using the STATISTICA v. 6.0 (StatSoft Inc., Tulxa, UK) software.

3 Results and Discussion

3.1 Evaluation of the disease incidence of *B. cinerea*

Visual evaluation of the *B. cinerea* presence on berries was performed after a 26 days cold storage period. The average disease incidence (DI) for all the three treatments was calculated (1) to assess the efficacy of ozonated water against the grey mould during the cold storage period.

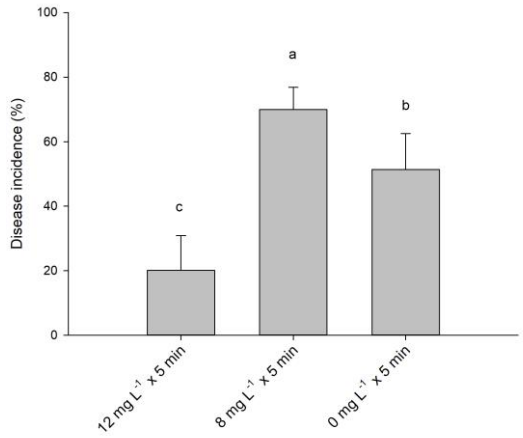

Figure 3. Disease incidence (DI) evaluation results. Data are shown as a mean of eight replicates with standard deviation (vertical bars). Bars labeled by different letters are significantly different according to the Tukey's test (p <0.05).

Untreated berries showed an average disease incidence of 51.4% (Fig. 3). The application of two different ozone concentration in aqueous phase expressed statistically relevant differences compared to the control. In particular, the berries treated with 12 mg L^{-1} ozonated water showed a significant disease incidence reduction of 61.0%, compared to the control. On the contrary, berries treated with 8 mg L^{-1} ozonated water, showed a significant disease incidence increment of 36.0% compared to the control.

3.2 Biological assessment of the berry microbiome

Biological assays on berries treated with two different ozonated water concentrations (12 mg L^{-1}, 8 mg L^{-1}) were performed to investigate the effect of ozone against grape epiphytic microbiome. The microbial count was conducted on three different selective solid media to define the general complexity of the microbial populations after the ozonated water treatments. Cell counts were executed serially diluting the mother solution three times up to a 10^{-3} CFU mL^{-1} concentrations, with the visual assessment of the bacterial, yeast and fungal colonies resulting coherent with the dilution volumes.

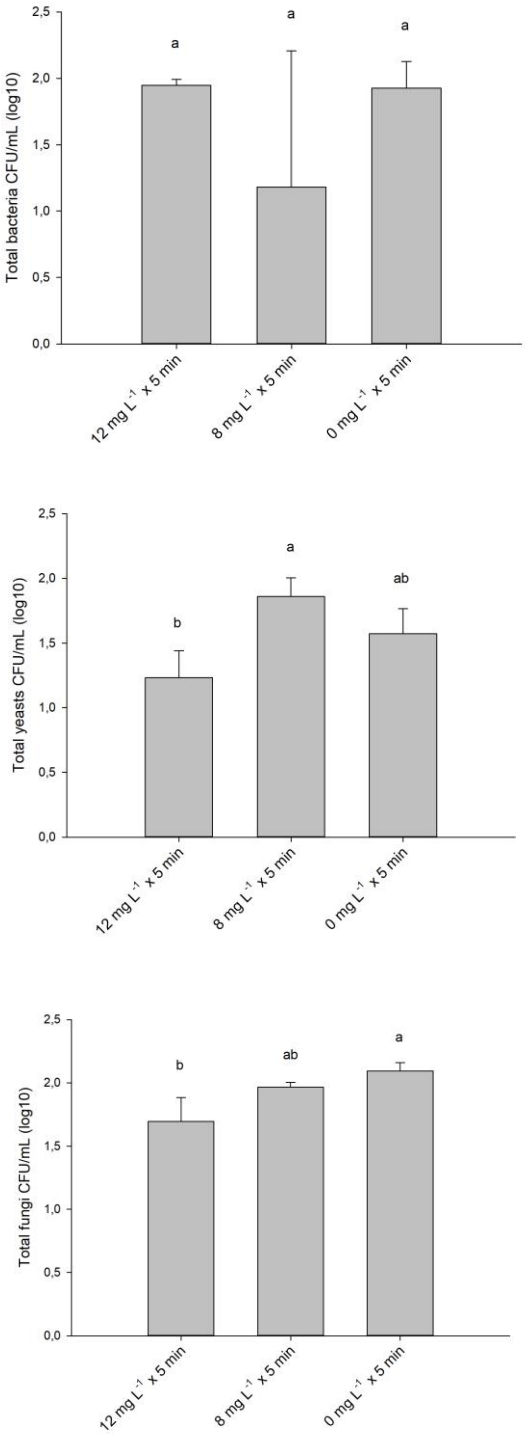

Figure 4. Biological assessment results of total bacteria, total yeasts and total fungi. Data are shown as a mean of three replicates with standard deviation (vertical bars). Bars labeled by different letters are significantly different according to the Tukey's test (p <0.05).

Differences between the treatments and the control in terms of reduction of the microbiome were assessed. After 26 days of cold storage the population of yeast and fungi on untreated berries reached 40.0 CFU mL^{-1} and 1.25×10^2 CFU mL^{-1}, respectively (Fig. 4). Only T1 managed to significantly reduce yeast and fungi populations (-54% and -58%, respectively) compared to the control, while no significant effects were found for bacteria population.

No effect of the treatments on bacterial population was assessed compared to the control. This result is in line with the current knowledge regarding the better resistance of bacterial spores against ozone [31].

4 Conclusions

Ozone is a GRAS-type decontaminating gas with marked oxidative activity that constantly gained more interest as a potential alternative, more sustainable mean to control fruit spoilage due to microbial pathogens, both in field and during post-harvest. Many studies already focused on the application of this sanitizing agent in its gaseous form, so the goal of this study was to provide more data on the effects of ozonated water during the post-harvest in semi-commercial conditions, to discuss potential critical points in its applicability.

From the results obtained in our experiments, performed on ready-to-eat table grape in semi-commercial conditions, it could be deduced that 12 mg L^1 ozonated water was the most efficient treatment, that significantly reduced both the grey mould and microbial populations of the berries, in terms of fungal and yeast populations. This result supports the positive impact of ozone treatments in more sustainable agronomical practices.

However, further experimental activities are needed to test a wider range of concentrations. In particular, testing concentrations between 12 and 8 mg L^{-1} could allow to identify efficient concentrations more economically friendly. Moreover, testing concentrations higher than 12 mg L^{-1} could be useful both to confirm the efficacy of higher ozone's concentrations in water and to assess any trend reversal of its activity against berry microbiome. Finally, another potential critical point not to underestimate is the quality of water used to dissolve the ozone molecules. It is known, in fact, that the interaction between ozone molecules diluted in tap water or water for food industry with organic substance potentially suspended in it, could interfere with the overall efficiency of ozone oxidative effects [31,32]. For this reason, in further studies using deionized water could allow to reach the desired ozone's concentrations faster, resulting in less energy expenditure of the overall process.

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