http://dx.doi.org/10.1080/01919512.2015.1074856

Ozone Effects on *Botrytis cinerea* Conidia using a Bubble Column: Germination Inactivation and Membrane Phospholipids Oxidation

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ABSTRACT

Ozone treatments were applied on conidia aqueous suspensions in order to determine the minimal applied ozone dose to limit conidia germination and to observe the mechanisms involved in the spores inactivation. Conidia germination was significantly reduced, bubbling for at least 0.5 min as a gas with a minimal ozone concentration of 1 $g.m^{-3}$. The applied ozone doses induce the membrane phospholipids oxidation, determined by the malondialdehyde quantification. Membrane phospholipids oxidation and inactivation rate are correlated. So, lipid peroxidation and consequently the alteration of the membrane integrity are involved in the antifungal action of ozone.

KEYWORDS

Ozone; *Botrytis cinerea*; Bubble Column; conidia; Germination; Lipid Peroxidation

Introduction

Gray mold is considered as one of the most important postharvest decays of the fruits throughout the world. It contaminates more than 200 different plants species (Govrin and Levine 2002). For example, it can be found on strawberries, blackberries or grapes (Archbold et al. 1997). Economic losses due to this mold are estimated about 2 billion dollars per year in the viticulture world (Ajouz 2009). In the case of kiwi fruits, percentage yield losses are usually between 0.2 and 2.0% but can reach up to 20% (Minas et al. 2010). The origin of this problem is the ascomycete *Botrytis cinerea*. This fungus has a dissemination form, the conidia, which is an important inoculum source of the disease. They are produced in the spring in field conditions but can be found throughout the year in sheltered spaces (Ajouz 2009).

Controlling this fungus is generally managed by pre- and postharvest fungicide treatments and can be completed by cultural methods (Minas et al. 2010). Recently, field isolates have developed resistance to boscalid and pyraclastrobin, two major fungicides used against this pathogen. Its control could become a major problem for many crops into the world (Bardas et al. 2010). Moreover, the fungicides used are both environmental and social concerns (Fujiwara, Hayashi, and Park 2011; Zhang et al. 2013). Indeed, chemical residues may cause the formation of toxic and carcinogenic organic compounds. So, developing new solutions to limit the development of pathogens represents a challenge (Sharpe et al. 2009).

Ozone could be an interesting alternative to fungicides to decrease the fungal pressure. Indeed, this strong oxidant is already used to clean drinking water, industrial wastewater or food-process equipment (Park et al. 2008). One of the main interests of ozone is its low remanence in the environment compared to conventional fungicides. When ozone is dissolved in water at 20 °C, its half-life is estimated between 20 and 30 min (Khadre, Yousef, and Kim 2001). Ozone has the advantage of decomposing spontaneously into nontoxic products, mainly oxygen (Sharpe et al. 2009). Ozone gas or ozone dissolved in water (called "ozonated water" for convenience) is efficient in eliminating bacteria, fungi or viruses (Guzel-Seydim, Greene, and Seydim 2004; Sands et al. 2004). Many studies have been done on the impact of ozone treatments on *Botrytis cinerea*.

Generally, ozone was applied in gas phase (Gabler et al. 2010; Liew, Prange, and Agriculture Canada 2000; Nadas, Olmo, and Garcia 2003; Ozkan, Smilanick, and Karabulut 2011; Palou et al. 2002; Sharpe et al. 2009; Tzortzakis, Singleton, and Barnes 2007). For example, enriching the atmosphere of a

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conventional cold storage room with gaseous ozone limits the germination rate of *Botrytis cinerea* conidia on artificially inoculated kiwi fruits within hours (Minas et al. 2010). However, applying ozone by gas exposure requires a structure with storage chamber or closed compartment. Moreover, Zorlugenç et al. (2008) demonstrated that aqueous ozone is more efficient against fungi than gaseous ozone. In their conditions, applying ozonated water for 7.5 min on dried figs reduced the total mold counts by 1.73 log CFU/g, whereas the reduction recorded with gaseous ozone during the same period was 0.59 log CFU/g. It is possible to dip vegetables (fresh cut lettuce and green bell pepper) in continuously ozonated water (Alexopoulos et al. 2013). This soaking decreases the microbial population (aerobic mesophiles, coliforms, yeast and molds) about 2 log CFU/g during the first 15 min and about 3.5 log CFU/g after 30-min exposure. Smilanick, Margosan, and Mlikota Gabler (2002) have tested the effect of the dipping into ozonated water (10 ppm) of table grapes contaminated artificially by *B. cinerea* spores. A 1-min dipping led to a 35% reduction of the gray mold development. However, the more organic material present in the treated solution, the more the ozone concentration is reduced (Khadre, Yousef, and Kim 2001). That is why the complexity of the studied media (conidia on fruits) can limit the ozone efficiency and does not allow the evaluation of the impact of ozone on *B. cinerea* spores germination.

Inactivation of the microorganisms by ozone is caused by the progressive oxidation of vital cellular compounds (Guzel-Seydim, Greene, and Seydim 2004). The primary target of ozone is cell surface and its integrity (Komanapalli and Lau 1996; Makky et al. 2011; Miller, Silva, and Brandão 2013; Scott and Lesher 1962). Ozone could act first on polyunsaturated fatty acids leading to the alteration of the membrane. Lipid peroxidation leads to a chain reaction from polyunsaturated fatty acids (Iriti and Faoro 2007) to malondialdehyde (MDA). The molecular formula of MDA is $CH_2(CHO)_2$. It is obtained during the cleavage of the polyunsaturated fatty acids having at least three double bonds (Lefèvre et al. 1998). Malondialdehyde forms, together with thiobarbituric acid (TBA), a pink-colored MDA-TBA adduct (Cho et al. 2010). This lipid peroxidation by ozone has been described on bacteria (Cho et al. 2010) and micro-alga (Wu et al. 2011) but, to our knowledge, never on fungi.

Thereby, ozone could be an alternative treatment against *Botrytis cinerea*. Several publications using ozone gas show the efficiency against this fungus. Ozonated water better limits the fungi development than does gas exposure. Some studies were done by washing or dipping inoculated fruits into ozonated water. This work presents, for the first time, an *in vitro* test to study the ozone action on *B. cinerea* spores without interactions with fruit support. The model proposed in this study is intended to confirm the fungicidal action and to evaluate the minimum applied ozone dose against spores. This study also aims to understand the action of ozone on conidia membrane thanks to MDA tests.

Materials and methods

Cultures and conidia suspensions

Botrytis cinerea (CBS 126.58) was cultivated at 21 °C on Potato Dextrose Agar. *Botrytis cinerea* conidia aqueous suspensions were done with cultures 3 weeks old. Plugs of inoculated PDA were put in sterile distilled water and shaken in order to detach spores from mycelium. A number of shaking and/or addition of inoculated PDA plugs were done until about 1.0×10^5 spores/mL were obtained. The plugs were discarded and the final concentration was assessed using a Malassez cell. pH of the spores suspension was about 7.3.

In vitro test of ozone

Ozone was produced with a LAB2B Ozone Generator (Ozonia, Dubendorf, Switzerland) supplied by an oxygen bottle for the concentrations higher than 10 $g.m^{-3}$ and a nitrogen/oxygen mixture (78.09/20.94% vol.) for the concentrations of 1 or 2 $g.m^{-3}$. The inlet ozone concentration was controlled by an ozone analyzer BMT 961 (BMT, Berlin, Germany). Ozone gas was bubbled in 10 mL of *Botrytis cinerea* conidia aqueous suspensions to obtain a constant ozone concentration dissolved in the water (Figure 1). The gas flow rate was fixed at 10 $L.h^{-1}$.

Several modalities were tested. Two repetitions were done by modality. The ozone concentrations in inlet gas were 1, 2, 10, 20 and 30 $g.m^{-3}$. The bubbling has been applied for 0.5, 2.0, 4.0 and 5.0 min. The applied ozone dose on spores solutions can be estimated by using the following equation:

$$\frac{[O_3]_g \times T \times D}{60 \times V} = \text{applied ozone dose}$$

where $[O_3]_g$ is the ozone concentration in inlet gas in $g.m^{-3}$; T is the duration of ozone application in min, D is the flow rate in $L.h^{-1}$ and V is the volume of the solution in mL (which is equal to 10 mL). The dose is expressed in $mg.mL^{-1}$.

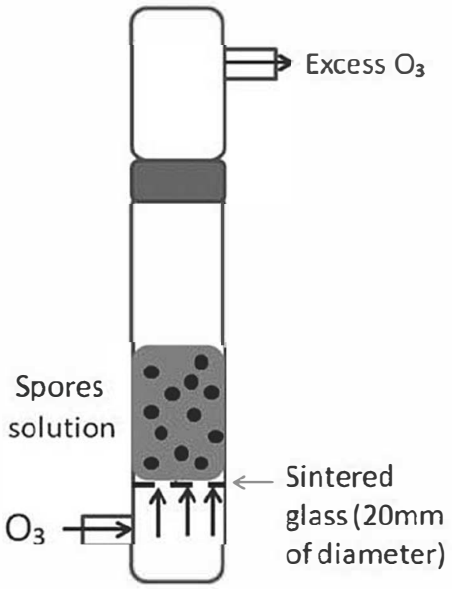

Figure 1. Schematic diagram of the 25-ml experimental reactor for the ozone application.

The different doses applied in this study are presented in the Table 1 modality by modality.

By dividing the ozone dose by the amount of spores present in solution, it is possible to determine the ozone dose (in mg) used by spore.

Effect of ozone on conidia viability

Evaluation of the germination rate

The germination rate was determined by counting the germinated spores in the water. That is why 20 µL of untreated or treated *Botrytis cinerea* conidia aqueous suspensions were placed on a Malassez cell counting chamber. Counting chamber was introduced in a high humidity compartment (at least 90%). The compartment was conserved at 21 °C with 14 h of daylight for 48 h. After this period, the germination rate was evaluated by observation under an optical microscope (Leica DM750 light microscope, Leica Microsystems GmbH, Germany). Then, 100 conidia were randomly taken. When a germ tube was present and measured more than 3 µm, the spore was considered germinated. The germination rate was calculated using the following equation:

$$\text{Germination Rate} = \frac{\text{Number of germinated conidia}}{\text{Total of counted conidia}} \times 100$$

Four countings were done by modality.

Table 1. Applied modalities and their equivalent doses (in mg. mL ^{1} of ozone) for a flow rate of 10 L.h ^{1}.

	Modality	Ozone concentration in inlet gas ($g.m^{-3}$)	Duration of bubbling (min)	Applied ozone dose ($mg.mL^{-1}$)
High Ozone Concentrations Modalities	0a	0	0	0.0
	0a'	0	4	0.0
	1	10	0.5	0.08
	2	20	0.5	0.2
	3	30	0.5	0.3
	4	10	2	0.3
	5	20	2	0.7
	6	30	2	1.0
	7	20	5	1.7
Low Ozone Concentrations Modalities	0 b	0	0	0.0
	8	1	0.5	0.01
	9	1	1.0	0.02
	10	1	2.0	0.03
	11	1	4.0	0.07
	12	2	0.5	0.02
	13	2	2	0.07
	14	2	4	0.1
	15	2	5	0.2

Assessment of biocide character

For the modalities 0a, 2, 5 and 7, a monitoring was realized on Petri dishes. After the bubbling, 100 µL of the different solutions were sprayed on PDA by using a Drigalsky spatula (two dishes were done per dilution). During the incubation, dishes were stored in the same conditions (at 21 °C with 14 h of daylight) as the compartment carrying the Malassez cells. The Petri dishes were observed after 5 and 28 days to compare the development of fungal spots. Three repetitions were done by modality.

Lipid peroxidation

After the ozone bubbling into the spores solutions, these solutions were concentrated by centrifugation about 1.2×10^{6} spores in 1 milliliter. Each sample was sonicated (VibraCell 75185, Sonics and Materials, USA) for 5 seconds. The quantity of MDA produced was quantified using a OXltek TBARS Assay Kit (Enzo, ALX-850-287). Briefly, after the addition of SDS and the TBA/Buffer reagent, the incubation at 95 °C for 60 min and cooling to room temperature, samples were centrifuged, and the supernatant was removed for analysis. The absorbance was measured at 532 nm (spectrophotometer Sunrise, Tecan, France). The calibration was done using a MDA standards curve. The concentration of MDA is expressed in nmol/mL.

Data analyses

Mean values of germination rates were compared using the Kruskal–Wallis test ($\alpha = 5\%$). Applied modalities of ozone concentration and duration were used to build a response surface.

MDA concentrations were compared by Tukey's test (α = 5%) after an analysis of variance via Xl-Stat software.

Results

Effect of ozone on conidia viability

Evaluation of the germination rate

In the first step, reactor capacity to spread ozone and the ozone ability to impact the viability of conidia were tested.

The conidia germination is about 80.0% without any treatment (modality 0a), whereas germination rates of the ozonated solutions (modalities 1, 2, 3, 5 and 7) with high ozone concentrations are lower than 3.0% (Figure 2). 0a' attests that the antifungal effect is caused by ozone application and not by the bubbling process; the germination rate of 0a' is actually about 98.0%.

So, ozone treatments significantly ($p < 0.05$) inactivate spores' germination as compared to the nontreated samples. In the case of high concentrations, ozone application induces an inhibition of the germination, even if the dose is low (0.08 mg.mL ^{1} for the modality 1) and applying a short duration (0.5 min) is sufficient.

In a second step, tests were done to bring out the minimal applied ozone dose of ozone. Experiments were carried out with low ozone concentrations to identify the limit of the antifungal action of the treatment. The ozone concentrations applied were 1 and 2 g.m ^{3} for 0.5, 2.0 and 4.0 min. Figure 3 shows that the germination rate of the control (modality 0b) is 80.0% and is close to the germination rate of the previous control sample which was 80.0% too (modality 0a). Under the conditions of the study, the minimum effective dose against *Botrytis cinerea* conidia is about 0.07 mg.mL ^{1} corresponding to 7.0 × 10 ^{7} mg of ozone/spore. The fungicide action disappears when the bubbling is only maintained for 0.5 min with a concentration of 1 or 2 g.m ^{3}. In this case, the treatment is ineffective. However, increasing the period of bubbling improves the efficiency of the treatment. For a given dose (modalities 9 and 12 or modalities 11 and 13), the duration seems to have a real importance (even if no significant difference is recorded). That is why the response surface of the viability in terms of the concentrations and durations applied is presented in the Figure 4. Indeed, germination rate can be modelled by the following equation:

$$\text{Germination Rate} = 97.4 - 9.2[O_3]_g - 26.3T + 2.8[O_3]_g \times T$$

where $[O_3]_g$ is the ozone concentration in inlet gas in g.m ^{3} and T is the duration of ozone application in min. The germination rate is in %.

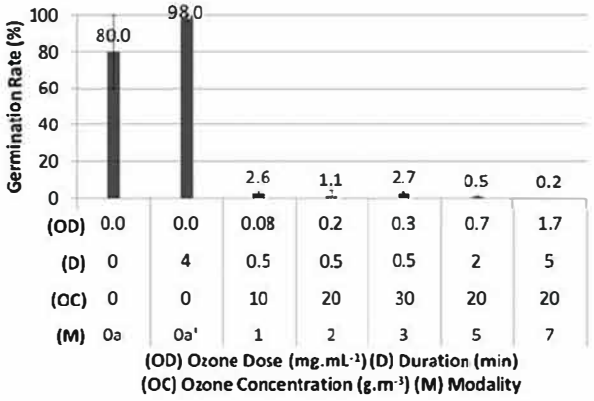

Figure 2. Germination rates of non-treated samples and treated samples for "high ozone concentrations modalities."

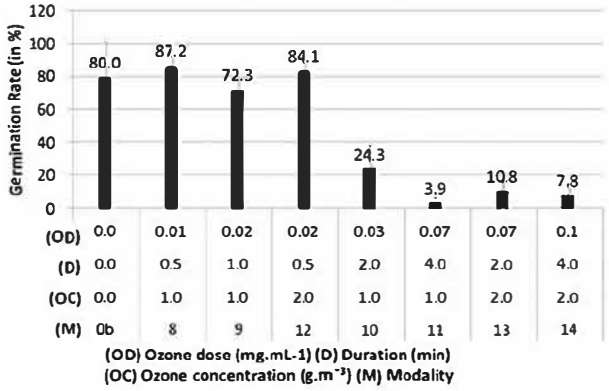

Figure 3. Germination rates of non-treated samples and treated samples for "low ozone concentrations modalities".

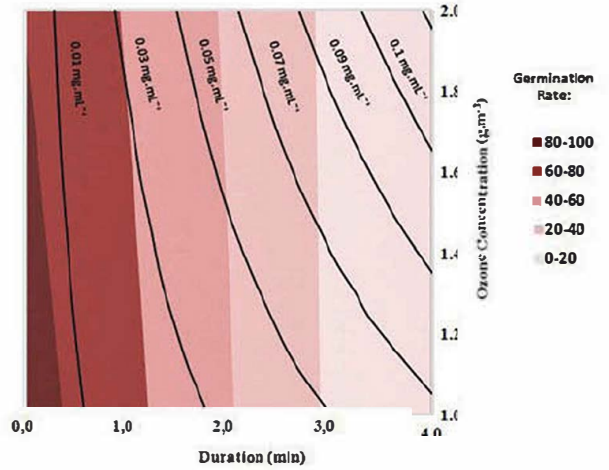

Figure 4. Surface response for germination rate in the case of low concentrations (between 0 and 2 g.m ^{3}) between 0.0 and 4.0 min. The black lines represent the isodoses.

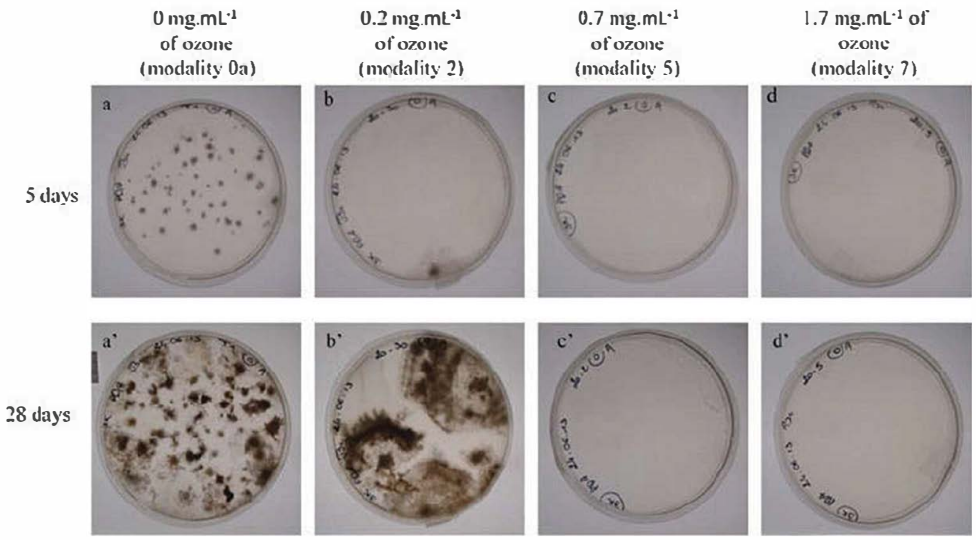

Figure 5. B. cinerea development on PDA from conidia solutions after 5 days (pictures a, b, c and d) and 28 days (pictures a', b', c' and d') in culture chamber.

Both for 1 and 2 g.m ^{3}, the longer the duration of ozone treatment, the less the conidia germinate. It brings out the case where the treatment is efficient. Beyond 0.07 mg.mL ^{1} isodose, the germination rate is lower than 40%. Above this isodose and with low ozone concentrations, increasing the duration is the best way to improve the fungicide action of ozone treatment.

Assessment of biocide character

The monitoring on Petri dishes was carried out in order to verify the biocide character of ozone treatment. The following pictures present the four modalities of the same repetition and dilution. They were taken 5 days after the inoculation (Figure 5, pictures a, b, c and d). We can note the absence of fungi on the two higher-treated dishes (modalities 5 and 7), whereas the non-treated dish (modality 0a) carries many fungal stains. Only one fungal spot grew on the intermediate dose (modality 2, picture b).

After 28 days in the culture chamber, the treated dishes count no fungus except the lower treatment (modality 2). In this case, about 3% of conidia have not been reached by the ozone. These few conidia grew and occupied the entire place 28 days later. The ozone bubbling seems to be a biocide and not a biostatic treatment. Indeed, the germination is definitively stopped thanks to the ozone treatment; no spore grew even after 28 days.

Lipid peroxidation

In this part, lipid peroxidation produced by ozone application was examined in order to be linked with the inactivation of spore germination. The MDA concentration increased with the rise of applied ozone doses (Figure 6). According to Tukey's test ($p < 0.05$), the untreated samples (modality 0) were significantly different to the treated modalities (modalities 4, 6, 13 and 15). The control samples recorded 3.9 nmol of MDA per mL. Among the treated modalities, three levels, between 8.0 and 21.3 nmol/mL, were recorded depending on the applied ozone doses. The more the applied ozone dose is important, the more the MDA concentration is high.

Germination rates versus MDA concentrations obtained during these experiments are represented in Figure 7. The more the MDA concentrations increased, the less high were the germination rates. The increase of MDA was directly correlated with spores' inactivation.

Discussion

Ozone is a strong oxidant that can limit the development of microorganisms (Khadre, Yousef, and Kim 2001). In this study, the bubbling reactor allowed us to work on spores solutions without interaction with fruits and to control exactly the duration of application and the ozone concentration. The germination of *Botrytis cinerea* conidia is inactivated by ozonated water. Raising the applied dose by extending the duration and/or concentration of treatment improves the antifungal action of ozone. These results are confirmed with Komanapalli and Lau (1996), who demonstrated that a short-term ozone exposure in aqueous solution can be insufficient to inactivate bacteria such as *Escherichia coli*, but increasing the duration of exposure

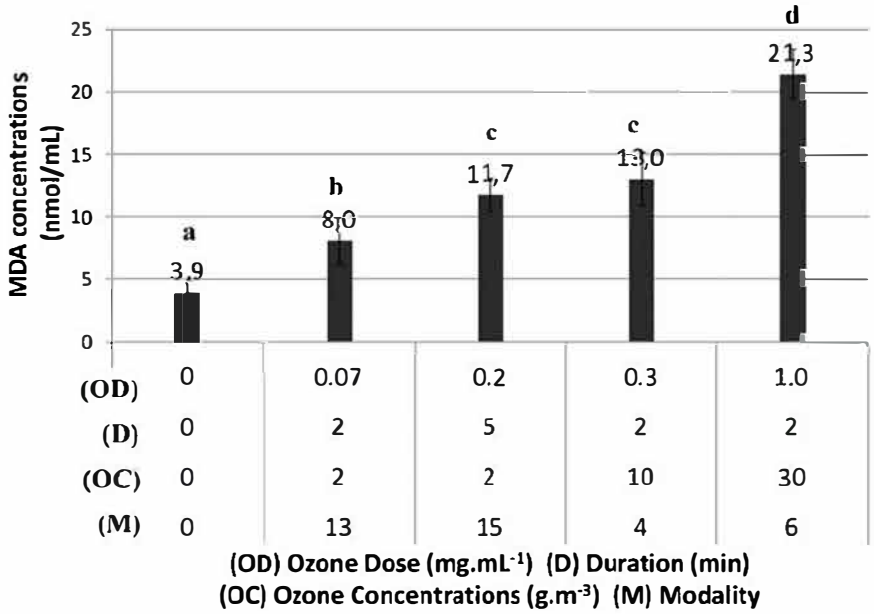

Figure 6. MDA concentrations (nmol/mL) in terms of ozone dose (mg.mL ^{1}). Letters indicate statistical differences according to Tuckey Test (α = 5%).

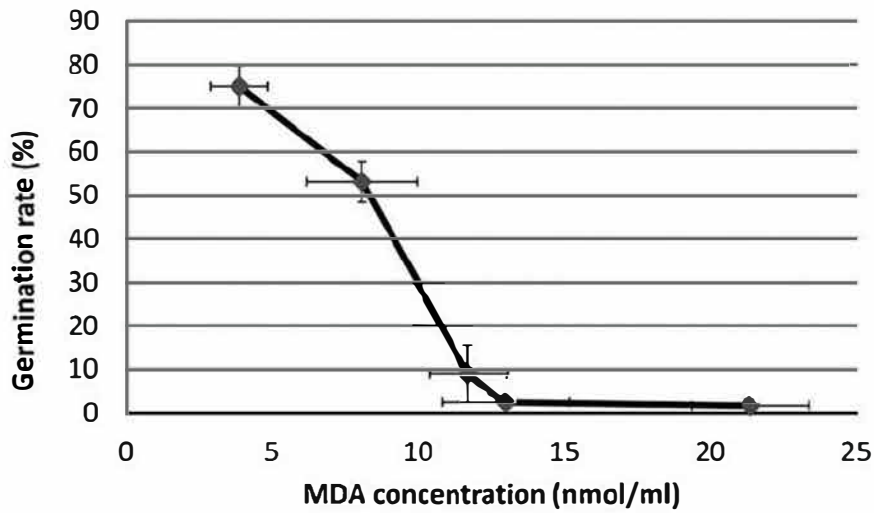

Figure 7. MDA concentrations (nmol/mL) in term of germination rate (%).

leads to decrease cell viability. In our study, the minimal dose to limit spores development is 7.0 × 10 ^{7} mg of ozone/spore. The minimal dose found in the literature to begin the inactivation of bacteria is less important (approximately 2.4 × 10 ^{7} mg of ozone/cell according Komanapalli and Lau (1996)). It can be explained by the fact that fungal spores are considered more resistant than bacteria (Restaino et al. 1995) probably because of the differences in the membrane structure (Alexopoulos et al. 2013).

For the low doses, it is preferable to increase the duration of treatment than to raise the concentration of ozone. This increase can allow the stabilization of the ozonation system and a better transfer of the gas into the water. Indeed, the amount of ozone transferred to the liquid increases with the gas-liquid contact time (Tapp and Rice 2012). This enhancement allows also raising the interaction between the constituents of the wall and the ozone. Finally, this study brings interesting information on the logic to be followed during *in vivo* tests. Nevertheless, the minimal concentration and time will be probably more important. Indeed, organic matter that have a high ozone demand can compete with spores for ozone treatment (Khadre, Yousef, and Kim 2001). Fungi can also penetrate in the fruit and be partially protected.

In this study, the MDA concentrations rose with the ozone doses. Moreover, it can be suggested that spores inactivation and lipid peroxidation were linked. The molecule of MDA is one of most profuse individual aldehyde compounds made after peroxidation of polyunsaturated phospholipids in lipid membrane (Cho et al. 2010). This phenomenon implies the alteration of membrane integrity (Iriti and Faoro 2007) and can be considered as sufficient to reach the cell lyses. So, lipid peroxidation is involved in the alteration of the cell membrane and could explain the antifungal activity of ozone.

To the best our knowledge, this is the first MDA test on fungal spores after ozone treatment. Assays were done on algae where the similar logic was recorded: the MDA concentrations rise with the ozone doses (Wu et al. 2011). In the case of bacteria inactivation, one of the mechanisms proposed is also the oxidation of unsaturated fatty acids by ozone (Cho et al. 2010; Iriti and Faoro 2007; Khadre, Yousef, and Kim 2001) but others' mechanisms could be implied. Some authors affirm that if the attack of the membrane does not lead to cell lysis, ozone can penetrate and react with enzymes, proteins and nucleic acids (Komanapalli et al. 1997; Miller, Silva, and Brandão 2013). A recent work on fungal spores has provided evidence that ozone could alter the ultrastructure of spores, resulting in disintegration of the cytoplasm and degradation of mitochondria (Ong and Ali 2015). Therefore, membrane degradation could just be one of the steps of the inactivation and not the only one. Moreover, two pathways can cause lipid peroxidation: the molecular ozone (called "direct reaction") or the hydroxyl radical (named "indirect reaction") pathways (Khadre, Yousef, and Kim 2001). In our study, pH of spore suspensions is about 7. The decomposition of ozone is not accelerated as would have been the case with high pH. The attack is probably mainly done by the molecular ozone pathway.

Finally, better understanding the fungicide action of ozone will provide critical information to determine disinfection strategies (Cho et al. 2010). *In vitro* data are a first step to anticipate *in vivo* results but need to be confirmed by *in vivo* tests (Ong and Ali 2015).

Conclusion

This study verifies the fungicidal effect of ozonated water against *Botrytis cinerea* conidia and proposes an *in vitro* model to identify the minimum applied ozone dose against this fungus. For the first time, the lipid peroxidation process was highlighted on fungus. It is possible to affirm that one of the mechanisms of the fungal inactivation by ozone is the alteration of the fungal membrane, leading to the spore inactivation. Measuring the MDA concentration could be an interesting tool to verify the ozone effect on other fungi. This *in vitro* model can be used to determine the minimal dose of ozone on other micro-organisms. It can also be a solution to continue the comprehension of mechanisms implied in the antifungal effect of ozone.

Acknowledgments

The authors thank Valerie Caffier for comments and fruitful discussions.

Funding

The authors are grateful to BPIFrance for their financial support (grant F1206003M) and Blue Whale SAS for their cooperation.

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