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## **The ozone molecule at the service of the vineyard**

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### **Summary**

**Keywords:** Three keywords separated by semi-colons. (Arial 10 pt, no indent)

The ozone molecule has proved its biocide properties over a wide range of microorganisms and applications. These biocide properties and the low remanence of the ozone molecule makes it a promising candidate to substitute controversial phytosanitary products used in agriculture. The present study aims to review several applications of the ozone molecule for its use in wine growing. From the nursery to the vineyard, different experimentations have been performed and have showed promising results: i) Ozone gas treatment for reduction of pathogens on crafted grapevines in nurseries, ii) irrigation with ozonated water for plant stimulation, iii) ozonated water for the esca treatment in nurseries and iv) ozonated water for the treatment of the downy mildew in the vineyard. For all these trials, the ozone molecule has proved its usefulness to replace or reduce the use of controversial chemicals in the wine growing.

### **Introduction**

The sector of the wine growing is facing a lot of challenges to make a transition toward practices more sustainable from an economical and environmental point of view (Chen, 2019). From the plant production in nurseries to the vineyards, operators are looking for innovative solutions to ensure the sanitary and the physiology quality of the vines. Along all the steps of production, controversial chemicals are often used to struggle against vines diseases and to improve their physiology (Pierron *et al.*, 2015). Those chemicals have high economical costs for the cultivators and high environmental and health impacts (Aubertot *et al.*, 2005). The regulation is putting the actors of the sector under pressure to make them replace the controversial phytosanitary products with solutions more environmental friendly (Guichard *et al.*, 2017).

The ozone molecule is a good candidate to substitute those chemicals since it has proved its efficiency to inhibit the development of a lot of microorganisms responsible of plants diseases with a low remanence on the treated matrix (Khadre, Yousef and Kim, 2001; Pagès *et al.*, 2016). Also several studies highlighted the ability of the ozone molecule to beneficially stimulate the plants physiology (Aziz *et al.*, 2003; Martínez-Sánchez and Aguayo, 2020).

In the present study, we propose a review of four different ozone applications for the wine growing sector at different steps from the nursery to the vineyard : i) Ozone gas treatment for reduction of pathogens on crafted grapevines in nurseries, ii) irrigation in nurseries with ozonated water for plant stimulation, iii) ozonated water for the esca treatment in nurseries and iv) ozonated water for the treatment of the downy mildew in the vineyard.

### **Materials and methods**

The main information about the methods used for each applications are summarized in table 1. Regarding the kind of applications (availability of closed spaces in the process, handling of the products, etc.), ozone have been applied in its gaseous form or dissolved in water (ozonated water). Most of the efficiencies of the ozone treatment are determined from a microbiological point of view : qualitative visual observations of the pathogens growth some days after the treatment or quantitative enumeration of the CFU of the pathogens after sampling of the product. Some physiological analyses have been performed such as roots and leaves length measurements to investigate the effect on the plant physiology.

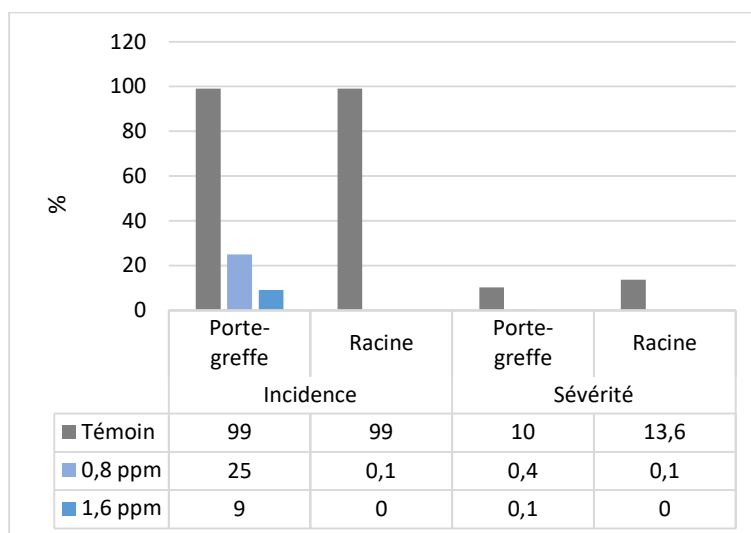
**Table 1.** Summary of the ozone treatment applied for the vines in the present paper.

Kind of application	Ozone treatment	Efficiency assessment
Ozone on crafted grapevines Nursery scale	Ozone gas	<ul style="list-style-type: none"> <li>• Visual observation of mycelial growth</li> <li>• Plant recovery</li> <li>• Mycelial quantification on roots</li> </ul>
Ozone for plant stimulation Nursery scale	Ozonated water irrigation	<ul style="list-style-type: none"> <li>• Roots development</li> <li>• Bud breaking quantification</li> <li>• Viability of the plant</li> </ul>
Ozone for esca treatment Nursery scale	Ozonated water	<ul style="list-style-type: none"> <li>• Spores observation</li> <li>• Spores germination</li> <li>• Gene regulation of the plant</li> </ul>
Downy mildew treatment Vineyard scale	Ozonated water spraying	<ul style="list-style-type: none"> <li>• Visual observations</li> </ul>

### Results and discussion

- Ozone on crafted grapevines

Visual observations of mycelial growth:

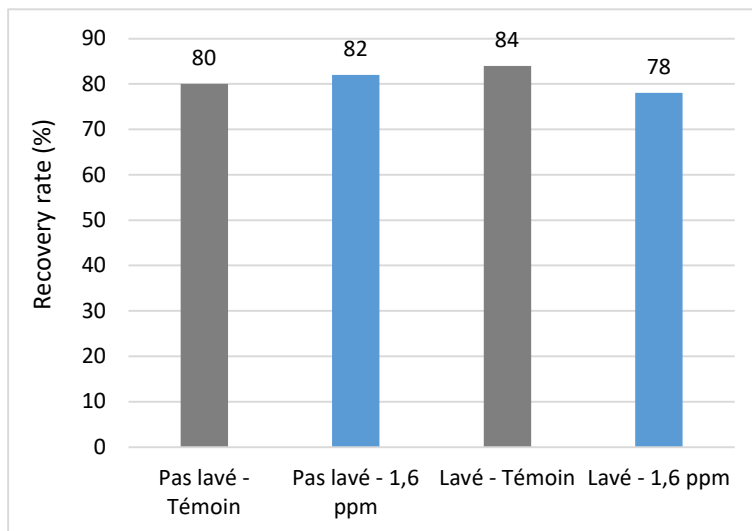


**Figure 1.** Percentage of trunk (porte-greffe) or roots (racines) that have shown mycelial development. Two ozone gas concentration have been tested (0.8 and 1.6ppm) and the mycelial grow have been measured in term of frequency (incidence) and intensity (sévérité).

After storage of the plants under the ozone atmosphere, visual observations were made to assess the development of the mycelial on the upper part of the plant and on its roots. It is clearly observable that ozonated plants show less fungal development than untreated plants.

To assess the frequency of fungal development, it is also important to notice that a higher concentration of ozone leads to a better efficiency of treatment.

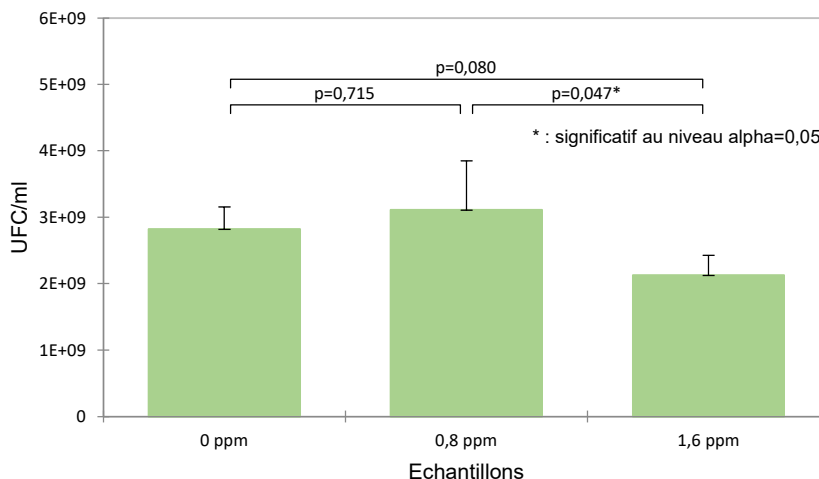
Plant recovery



**Figure 2.** Percentage of plants that have recovered after the planting process. A difference have been made between plants that have been washed or not before the ozone treatment (“lave” and “pas lave”). Blue bars represent the ozone treatments.

After treatment of ozone gas, the wood vines have been potted to observe day by day their ability to grow. Untreated and ozonated plants show similar recovery rates, meaning that the ozone treatment does not affect the growth of the plant after treatment. Nevertheless, even if the number of recovery plants is similar, the leaf development for the ozonated plants was visually better than untreated plants.

Mycelial quantification on roots



**Figure 3.** Counting of the UFC for mycelial quantification of the microorganisms that have infected the roots of the vines.

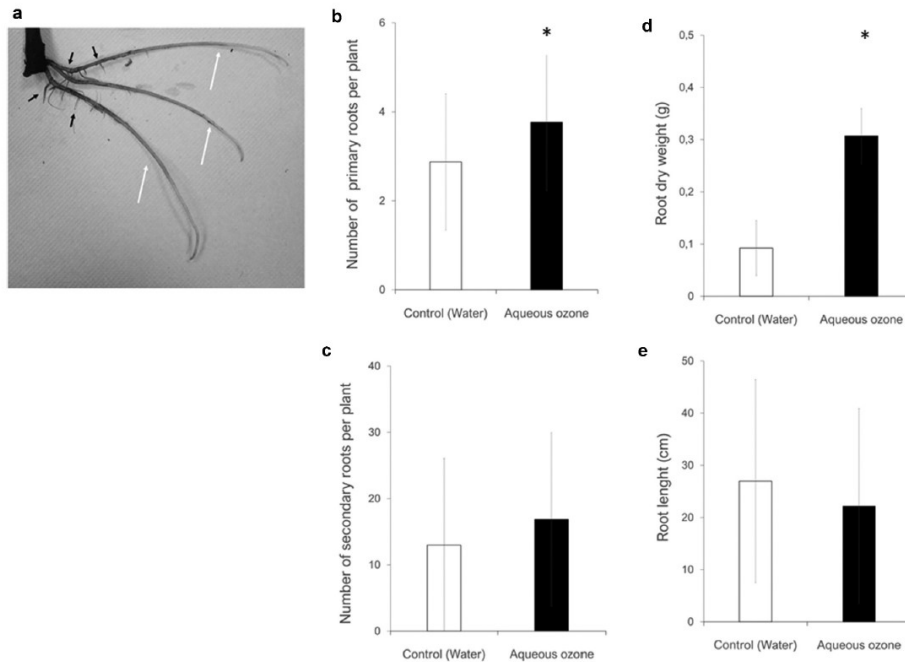
An attempt has been made to quantify the fungies on the roots of the plants after treatment. Results show that there is more or less the same fungies quantity on the plant but the visual observation above show different levels of infection.

- Ozone for plant stimulation

Root development:

After being irrigated with ozonated water, vines have been unpotted, and the number, the length and the weight of the roots have been measured. Results showed that there is a significant better

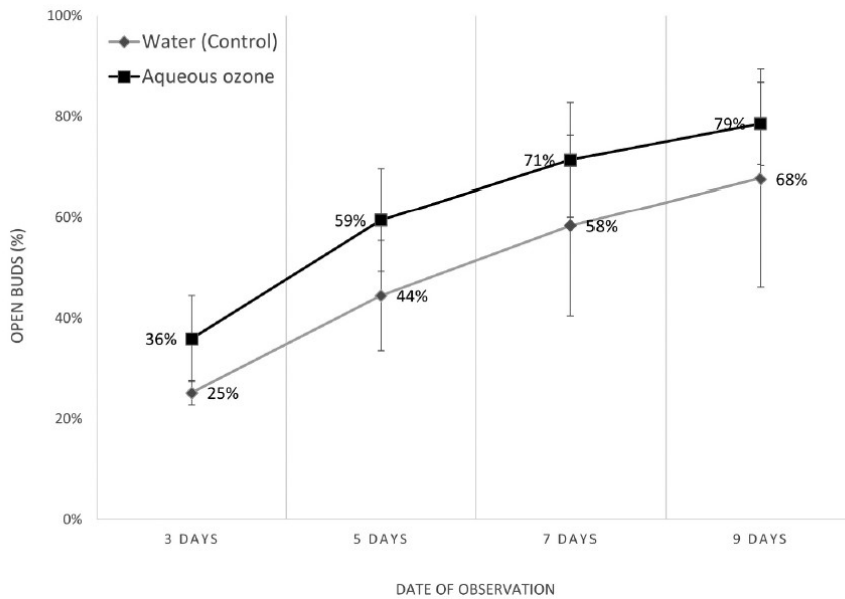
development of the roots of the plants since the number of primary roots and the mean root weight have increased for the ozonated plants.



**Figure 4.** (a) Primary roots (white arrows) and secondary roots (black arrows) of the root system of a 6-week-old grapevine rootstock plant. Comparison of the number of (b) primary and (c) secondary roots, (d) root dry weight and (e) root length of plants irrigated with aqueous ozone (black) or normal water (white). Asterisks represent significant differences between groups ( $p$ -value < 0.05) Error bars represent the SD ( $n = 3$ ).

**Bud breaking kinetics**

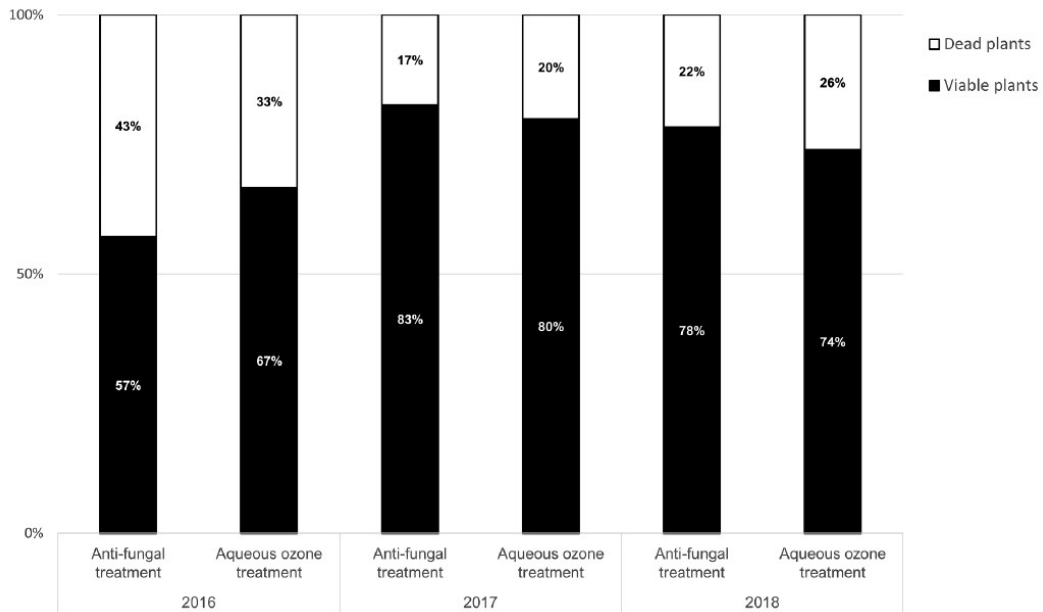
Also, during 10 days the plants have been observed in order to count the number of bud breaking (moment when the first leaf leaves the trunk). Although results showed incertitude of measurements, the mean trend seems to highlight that bud breaking occurs earlier with plants irrigated with ozone than the control ones.



**Figure 5.** Comparison of the percentage of open buds on plants irrigated with normal water (gray rhombus) or aqueous ozone (black squares) 3 days ( $p$ -value = 0.24), 5 days ( $p$ -value = 0.54), 7 days ( $p$ -value = 0.69), 9 days ( $p$ -value = 0.80) after the first budding occurred. This assay was repeated three times. Error bars represent the SD ( $n = 3$ ).

**Viability of the plants**

In order to assess the relevance of using an ozone treatment of the vines, the viability of the plants have been measured once the vines have been planted. The viability of the ozonated plants is compared with the conventional plant that have received a classical fungal treatment. Results showed that ozonated water allows to reach the same efficiency that the classical fungal treatments.

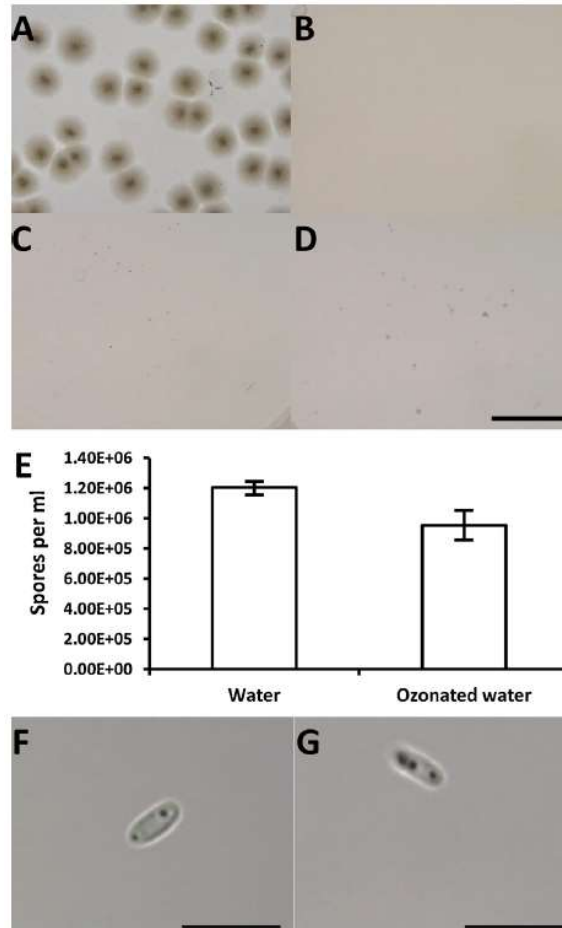


**Figure 6.** Comparison of the percentage of viable (black) or dead (white) plants at the end of the plant propagation process depending on the treatment followed (Aqueous ozone vs anti-fungal chemical treatment). The experience was performed three times, in 2016, 2017 and 2018. N=1000.

- Ozone for Esca treatment

Spores observation and quantification:

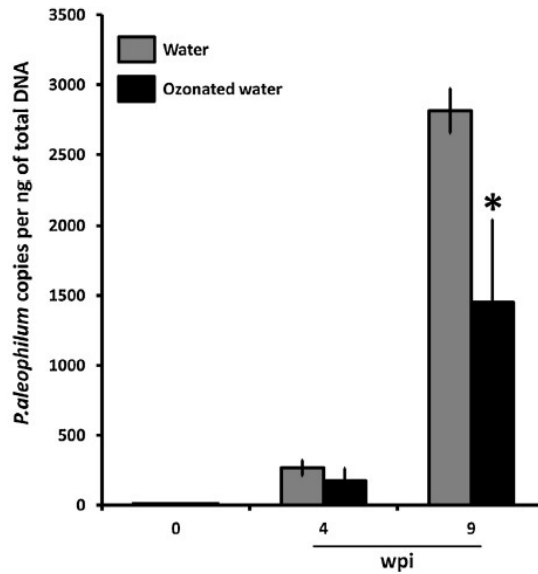
Once treated with ozonated water, spores solutions have been inseeded on petri dishes for visual observations and UFC counting. Also, microscopic observations were made to assess the integrity of the spores after the treatment. Results showed that ozonated spores are not able to develop and their quantity are lower in the solution. Microscopic observations seems to showed membrane degradation of the microorganism.



**Figure 7.** In vitro study of fungicide properties of ozonated water to control *P. aleophilum*. Photographs of young mycelia development of *P. aleophilum* spores treated with (A) sterile water, (B) 2.2 g/m<sup>3</sup>, (C) 4.5 g/m<sup>3</sup>, and (D) 13.5 g/m<sup>3</sup> of ozonated water (bar = 1 cm). (E) Number of spores per mL in suspension after treatment with water or ozonated water (13.5 g/m<sup>3</sup>); photographs presenting similar spore integrity after sterile (F) or ozonated (G) water treatment.

DNA quantification:

The part of the trunk of the vine that have been infected with the esca pathogens was collected to perform qPCR in order to have an idea of the living number of pathogens inside the trunk after the application of the ozone treatment. It has been clearly highlighted that the number of copies for the pathogen was lower in the trunk after the ozonated water treatment. It means that ozonated water treatment would inhibit the growth of the esca disease in the trunk when applied at the timing of infection.

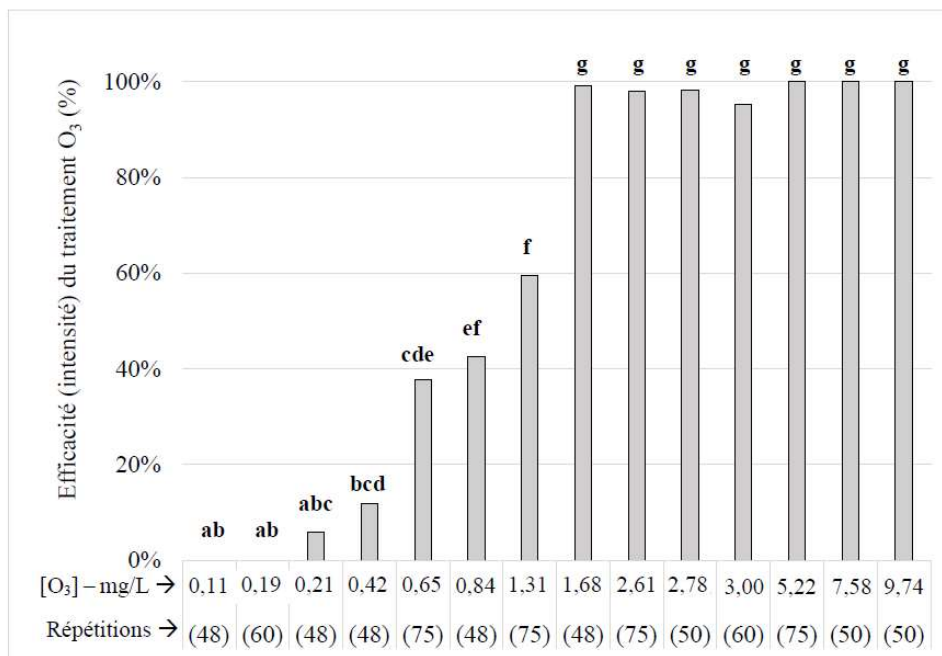


**Figure 8.** DNA quantification of *Phaeoacremonium aleophilum* 4 weeks (N = 6 samples of three pooled plants) and 9 weeks (N = 6 samples of three pooled plants) after treatment. DNA quantification reflects fungal development according to the two treatments: infected pruning wound receiving ozonated water (4.5 g/m<sup>3</sup>) or control infected injuries receiving sterile water. The time 0 corresponds to DNA quantification in non-treated plant material (N = 3). The asterisk (\*) indicates a significant difference as determined by the Kruskal–Wallis test (p value < 0.05) and wpi means “weeks post inoculation”.

- Ozone for downy mildew treatment

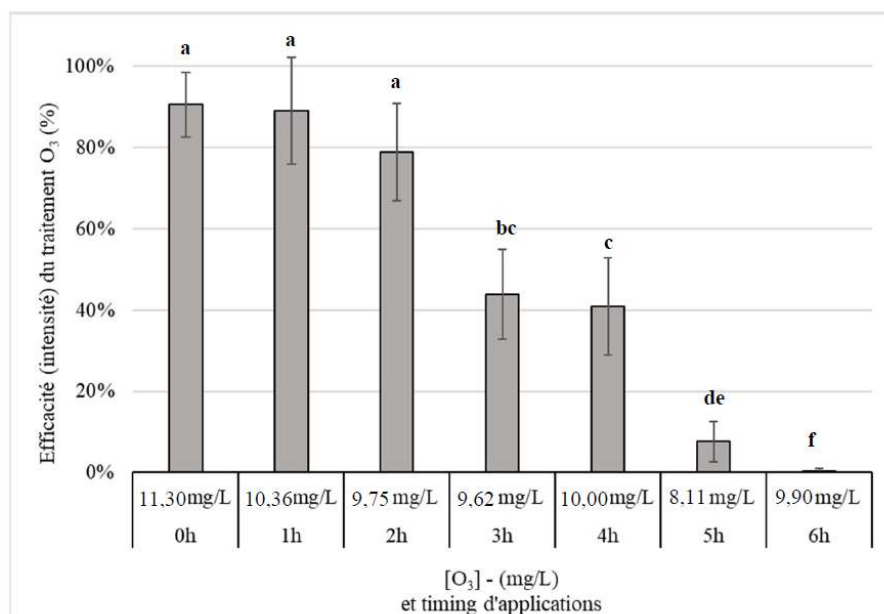
Ozonated water have been applied on downy mildew suspension at the in vitro and in planta level. Visual observations were made to assess the effect of the ozonated water on the pathogen development.

In vitro treatment:



**Figure 9.** Efficacy of ozonated water as a function of its dissolved ozone concentration in limiting the frequency of *P. viticola* sporulation after in vitro ozone/spore contact. The letters represent significant differences between the frequencies of each treatment modality according to the Kruskal-Wallis test (p-value < 0.05).

Ozonated water seems to inhibit the growth of the downy mildew when the applied concentration is around 2mg/L.

In planta treatment:

**Figure 10.** Effectiveness of in planta deposits of ozonated water in limiting sporulation intensity sporulation as a function of the time between inoculation with *P.viticola* sporangia and treatment (each modality corresponds to 3 independent experiments consisting of 48 notations each, i.e. n=144). The letters represent significant differences between the ratings of each treatment modality according to the Kruskal-Wallis test (p-value < 0.05).

In planta treatment allowed to understand that timing of application is of importance. Indeed, during its lifecycle, the downy mildew will develop inside or outside the leaf. Since the ozone has a “contact effect”, it cannot treat the downy mildew when it is inside the leaf. This information is important for the positioning of the treatment in real conditions.

### Conclusions

The present paper shows the relevance of the ozone molecule for the vines sector. It can be used at the nursery level to ensure a high quality of the plants, or during the growing phase to stimulate their growth. In the field, the ozone molecule can be used to perform crop protection and control the level of infection of the downy mildew.

Since the ozone molecule have shown efficiency on a wide range of pathogens, the present study that have been made here for the vine sector could be done for other sectors such as apples, pears, bananas, and so on, with the aim to replace controversial phytosanitary products.

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