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## Ozonated water to treat downy and powdery mildews in agriculture

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### Summary

Ozonated water (OW) 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 the OW a promising candidate to substitute controversial phytosanitary products used in agriculture. The present study aims to prove that ozonated water can inhibit the development of three mildews pathogens: *Plasmopara viticola*, the downy mildew of the vine; *Hyaloperonospora parasiticae*, the downy mildew of the cabbage; and *Oidium neolycopersici*, the powdery mildew of the tomato. Different levels of treatments have been applied: an “in vitro” treatment with high controlled conditions, an “in planta” treatment with moderated controlled conditions, and an “in situ” treatment mooking real treatments conditions by spraying. Note that, the efficiency of the ozonated water spraying has been improved by using a patented anti-desorption device based on recent studies. OW has been proved to be a promising alternative to treat those plant diseases.

**Keywords:** Pest management, *Plasmopara viticola*, *Hyaloperonospora parasiticae*, *Oidium neolycopersici*, pesticide alternative, dissolved ozone

### Introduction

Among the many diseases encountered in agriculture, mildews are one of the most devastating, implying high economic losses (Gessler et al., 2011). Downy and powdery mildews are two distinct plants diseases that can infect a lot of different kind of cultures and are provoked by microorganisms differing from a plant species to another (Burruano, 2000; Choi et al., 2012; Jones et al., 2001). Those diseases are nowadays handled by the spraying of copper-based, sulfur-based and chemicals-based solutions on the crops to inhibit their spread. These solutions are efficient but have raised multiple concerns from their extensive use : environmental pollution, health issues and pathogens resistance. Alternative solutions are needed to reduce the massive use of those controversial phytosanitary products (Dagostin et al., 2011).

The biocide properties of the OW has now been proved to be effective over a wide range of microorganisms and applications (Khadre et al., 2001). Ozone molecules dissolved in water have a short life time ( $\approx 20$ min) due to its self-decomposition. This self-decomposition leads, for most of the reaction pathways, to the formation of dioxygen (Staelin & Hoigne, 1985). Thus, its biocide properties, its low remanence and its decomposition in an harmless co-product, make the OW a promising candidate to substitute controversial phytosanitary products used in agriculture.

The present study aims to prove that ozonated water can inhibit the development of three mildews pathogens: *Plasmopara viticola*, the agent responsible for downy mildew in grapevine; *Hyaloperonospora parasiticae*, the agent for downy mildew in cabbage; and *Oidium neolycopersici*, the agent for powdery mildew in tomato. Different levels of treatments have been applied: an “in vitro” treatment with high controlled conditions, an “in planta” treatment with moderated controlled conditions, and an “in situ” treatment mooking real treatments conditions by spraying.

Note that a special attention has been payed when spraying ozonated water since ozone desorption occurs during the spraying (Canado et al., 2020). A patented device to efficiently spray ozonated water has been used.

## Materials and methods

- Vegetal materials

Vines have been grown from Merlot woods during 3 months in growing chambers with light and temperature regulations (14h/10h light cycle, 21°C and some humidifiers). Young leaves were then collected and cut with a cork-borer to produce 12mm diameter leaf discs. These discs were the vegetal supports for the development of the biotroph microorganism *Plasmopara viticola*.

Cabbage and tomato seedlings have been grown in greenhouse for 1 month with natural light cycles and temperature regulations.

- Pathogens inoculations

For the three pathogens, sporangia suspensions have been prepared by collecting sporulating structures of infected leaves. The spore concentration was assessed with an hemocytometer and then diluted to reach a concentration of 25 000sporangia/mL.

Inoculations on plants are made by droplets deposit on the vegetal support: each vine discs received 3 droplets of 20µL, each cabbage leaf received 2 droplets of 20µL, and each tomato leaf received 6 droplets of 20µL.

- Ozonated water preparation

Ozonated gas was produced with an ozone generator (ozonia CFS1 – Suez) between 10 and 100g/Nm<sup>3</sup>. Ozone gas was measured with an ozone analyzer (BMT 964 BT – BMT Messtechnik).

Ozonated water was procuded by dissolving the latter ozone gas in a 1.5L glass bottle. Dissolved ozone concentration applied for the treatments were determined by the indigo method just before their applications on the matrix of interest.

- Treatments

“*In vitro*” treatments consisted in putting in contact ozone molecules and pathogens sporangia before inoculations. In other words, sporangia are “ozonated” before being inoculated. For that, sporangia suspensions are diluted with ozonated water during the spore suspension preparation to reach 25 000 sporangia/mL.

“*In planta*” treatments consisted in placing a 20µL droplet of ozonated water with a pipette on the droplet of spore suspension previously inoculated on the leaf. Several ozonated water concentrations and timings between inoculation and treatments have been tested. Several inoculum loads have also been investigated.

“*In situ*” treatments consisted in spraying ozonated water with different concentrations on freshly inoculated vegetal materials at a distance about 40cm. As mentioned in the introduction, the spraying of ozonated water needs specific consideration to take into account to make an efficient treatment. A patented device has been used to perform such an efficient treatment.

- Visuals observation

The efficiency of the treatment with ozonated water has been assessed by visual observation of the pathogen development on the vegetal materials. For *P.viticola* and *O.neolycopersici*, each inoculations sites received a notation describing the sporulation of the pathogens. Comparison between the notation of control and treatments conditions allowed to define an efficiency of treatment parameter. Two efficiency parameters were defined : for the intensity and for the frequency of the infection :

$$Eff_{intensity} = 1 - \frac{\overline{N_{treatment}}}{\overline{N_{control}}}$$

$$Eff_{fréquence} = 1 - \frac{Freq_{treatment}}{Freq_{control}}$$

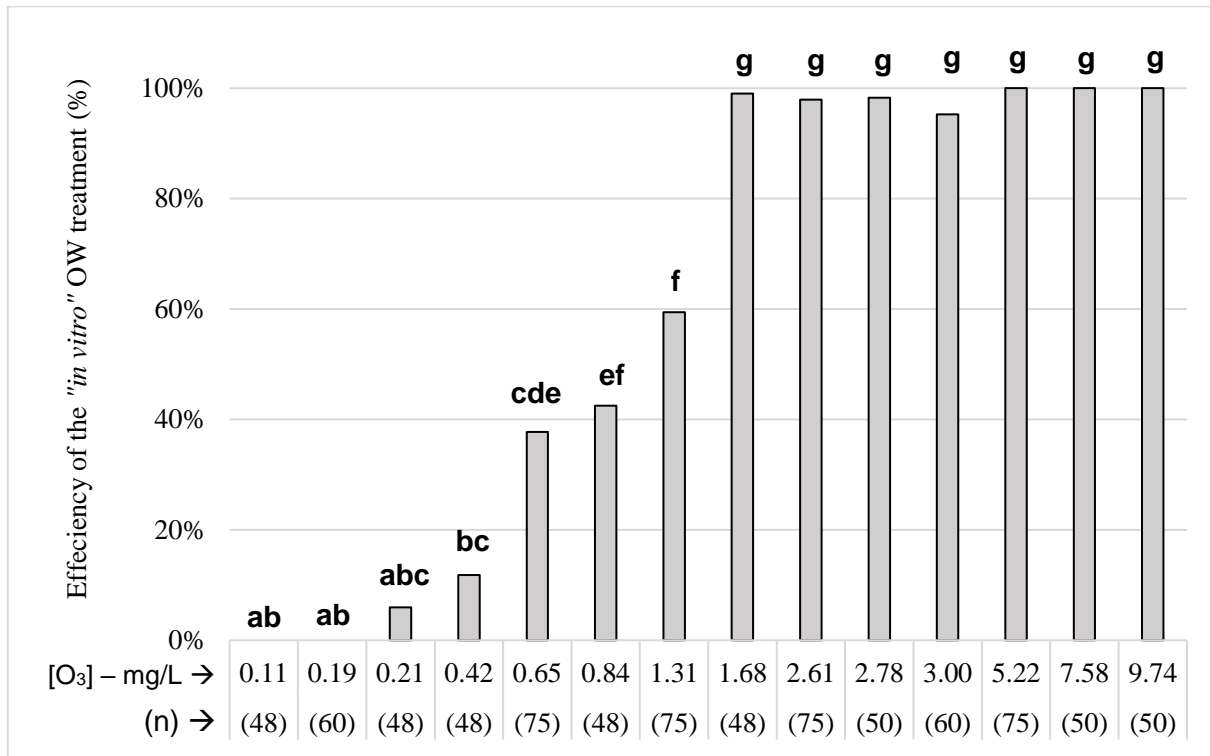
With  $\overline{N_{treatment}}$  and  $\overline{N_{control}}$  the average of the notations made respectively for the treatment and the control conditions;  $Freq_{treatment}$  and  $Freq_{control}$  the frequency of inoculations site showing a sporulation respectively for the treatment and the control conditions.

For *H.parasiticae*, the sporulation on the cabbage leaves was assessed using several classes of infection: Absence of symptoms (0), necrosis without sporulation signs (1), mild sporulation localized around the inoculation spot (3), mild sporulation on the two faces of the leaf (5), strong sporulation around the inoculation sport (7), strong sporulation on the two faces of the leaf (9).

### Results and discussion

- “In vitro” assays on *P.viticola*

“In vitro” treatments on the downy mildew of the vine showed a significant effect of the ozonated water to inhibit the development of the pathogen as plotted in figure 1.

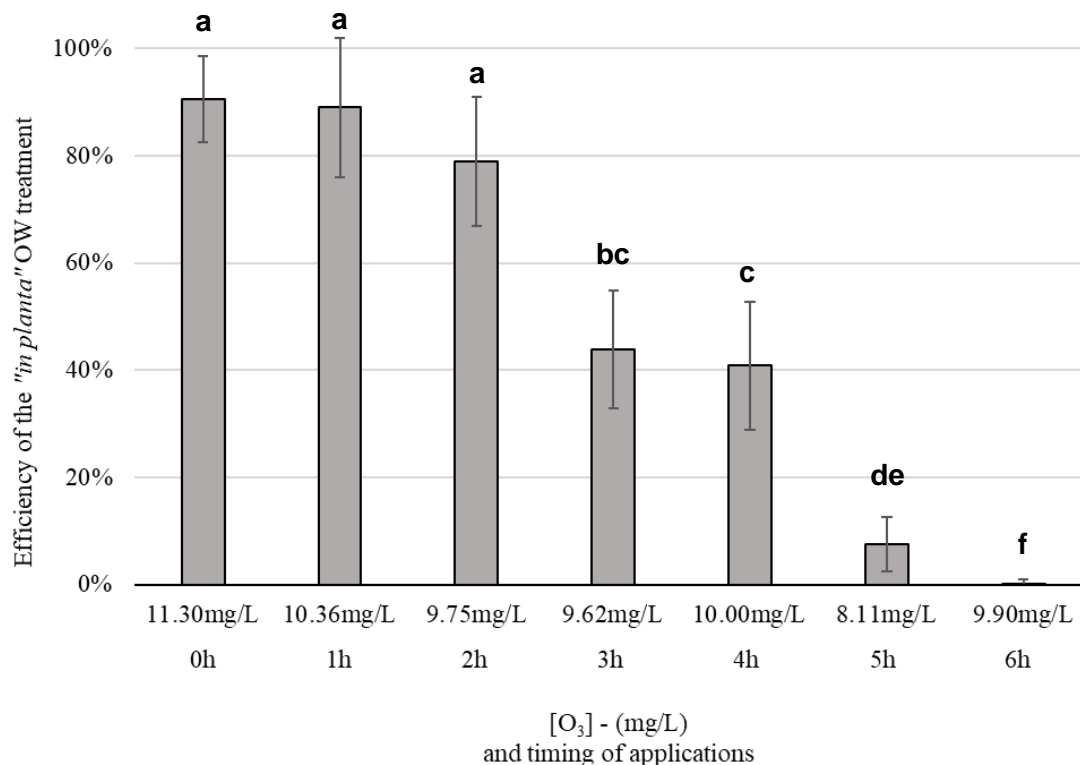


**Figure 1.** Efficiency of the ozonated water as a function of its concentration of dissolved ozone in limiting the intensity of sporulation of *P. viticola* after their contact *in vitro*. The letters represent significant differences between the ratings of each treatment modality according to the Kruskal-Wallis test (p-value < 0.05).

It shows that increasing concentration of dissolved ozone allowed to reach a higher efficiency of treatment. It can be explained by the fact that more ozone molecules are available to attack the membrane of the pathogen (Brodowska et al., 2018; Victorin, 1992). With an dissolved ozone concentration of 5.22mgO<sub>3</sub>/L, the growth of the *P.viticola*'s sporangia is completely prevented. It means that enough ozone has been brought in the sporangia solution to inactivate all the inoculum load of 25 000sporangia/mL and once the sporangia solution has been inoculated on the vines leaf discs, they were not able to infect and developed in the plant.

- “In planta” assays on *P.viticola*

“In planta” treatments with the downy mildew of the vine showed a significant effect of the timing of application of the ozonated water to inhibit the development of the pathogen as plotted in figure 2.

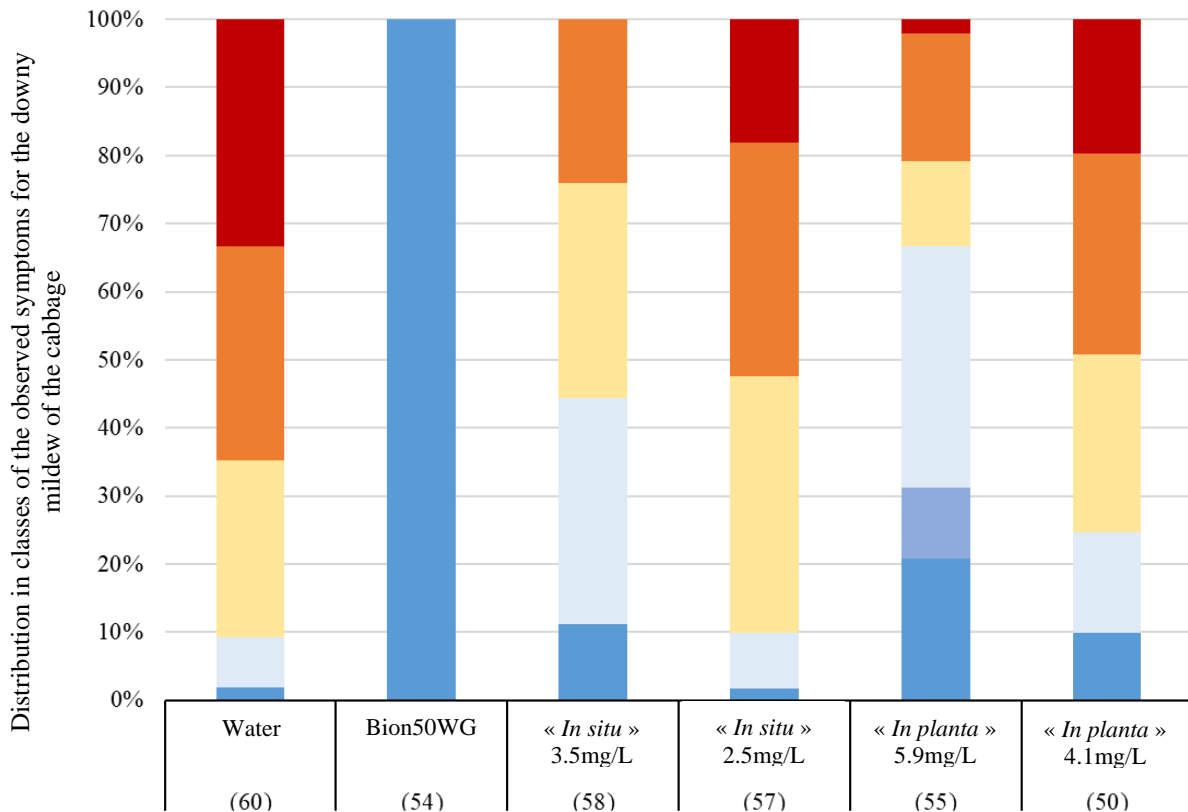


**Figure 2.** Efficiency of “in planta” ozonated water treatment to limit the sporulation intensity as a function of the duration between the inoculation of *P.viticola* sporangia and the OW treatment (each modality corresponds to 3 independent experiments consisting of 48 ratings each, i.e. n=144). The letters represent significant differences between the ratings of each treatment modality according to the Kruskal-Wallis test (p-value < 0.05).

These results are in accordance with the cycle of the downy mildew and the mechanism of action of the ozone molecule to inhibit the microorganism: after the inoculation, downy mildew sporangia remain on the surface of the leaf for maximum 3h, and after that, they enter the leaf through the stomata and start to develop a mycelium within its intercellular part (Gessler et al., 2011). After one week of development, hyphae with new sporangia come out from the stomata to present the sporangia at the surface of the leaf with the hope to be spread to new leaf for a secondary infection. It is known that the ozone molecule reacts quite fast and with a lot of compounds. It is then commonly accepted that the ozonated water has a “surface” effect but cannot reach the inside of the leaf, because the molecules are consumed mainly by the surface (Pirani, 2011). So the ozone molecule cannot reach the mycelium developed by the pathogen inside the leaf. That explains why 3 hours after the inoculation, the ozone is ineffective to inhibit the development of *P.viticola* sporangia for these “in planta” assays.

- “In planta” and “In situ” assays on *H.parasiticae* and *O.Neolycopersici*

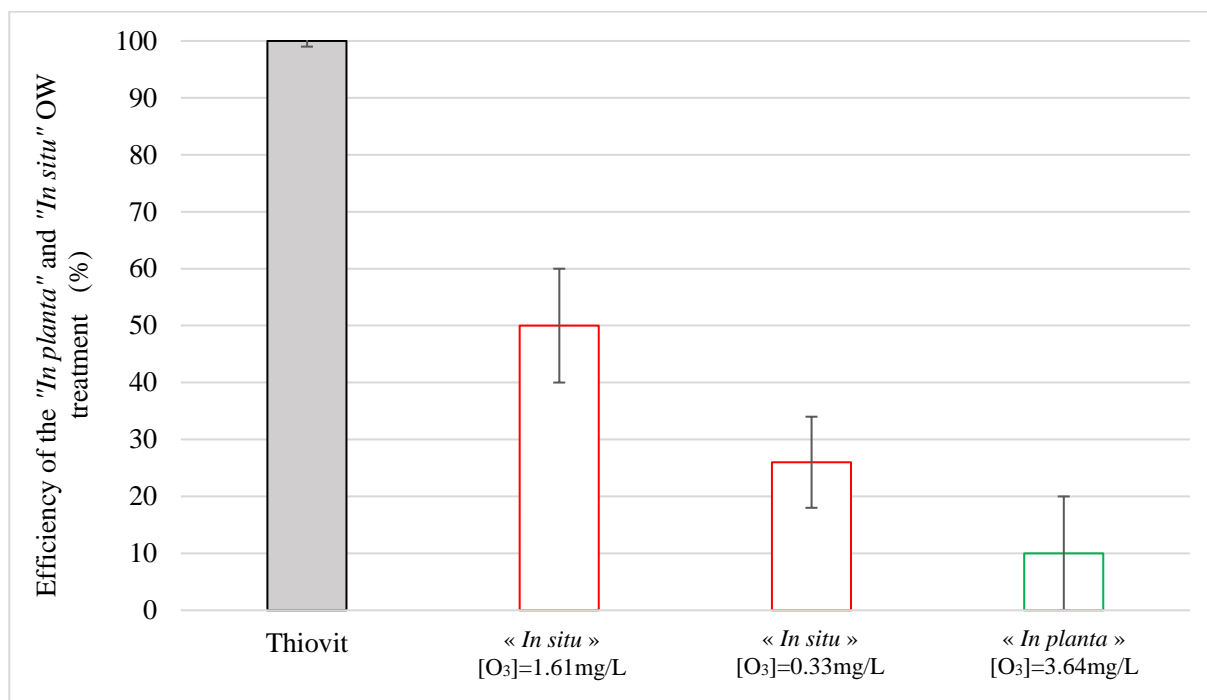
Figure 3 shows the repartition of the infection classes observed with *H.parasiticae* for each modality tested. It can be observed that the “in planta” treatment has reduced the number of cabbage leaves showing high degrees of infection (orange and red classes are lower than the control). It is important to note that the “in situ” treatment, by spraying, has been effective too to reduce the symptoms of the downy mildew of the cabbage. For each kind of treatment (“in planta” or “in situ”), better efficiency of treatment has been observed each time the OW concentration was increased as already observed with the “in vitro” assays. Note that the volume of OW applied was not the same between the “in planta” treatment (2 droplets of 20µL per leaf) and the “in situ” treatment (spraying until the leaves seemed correctly covered). That is why the efficiency of the treatment in terms of concentration applied cannot be compared for both treatments.



**Figure 3.** Distribution (in %) of cabbage seedlings in each class of downy mildew symptoms obtained after inoculation with *H. parasiticae*. The number in brackets indicates the number of notations made by modality.

Figure 4 represents the efficiency of the “*In planta*” and “*In situ*” treatments applied on the *O. Neolycopersici*, powdery mildew of the tomato. Once again, it is observable that treatments with OW have reduced the sporulation of the pathogen. As with the previous “*In planta*” assay, the development of the *O. neolycopersici* was not completely stopped since 100% of efficiency was not reached. But these treatments have the benefit of reducing the sporulating structures on the surface of the leaf which then reduce the potential of infection from a leaf to another when there are rainy and windy weathers.

So, “*In planta*” and “*In situ*” treatments showed a true effect of the application of ozonated water to reduce the development of mildews, for cabbage and tomato. It shows a certain universality of the ozone molecule to treat the wide class of mildews diseases.



**Figure 4.** Effectiveness of ozonated water treatments to reduce the intensity of sporulation of *O. neolycopersici* inoculation sites during an evaluation carried out 10 days after inoculation.

### Conclusions

Ozonated water applications just after inoculations (<4h) inhibit the development of *Plasmopara viticola*, *Hyaloperonospora parasiticae* and *Oidium neolycopersici*, common mildews diseases encountered in agriculture. Treatments efficiency increase with the dissolved concentration of ozone and by treating as close as possible of the inoculation moment. Ozonated water is then an innovative tool to substitute some controversial pesticides used in agriculture, with negative effects on the environment and health. It could be at least be used to reduce the potential of secondary infections of sporulating structures present on infected leaves.

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