In vitro and *in planta* fungicide properties of ozonated water against the esca-associated fungus *Phaeoacremonium aleophilum*

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ABSTRACT

Ozone could be used as a sanitary agent in the food and agricultural industries. The present study investigates whether ozonated water could be used to control conidia dispersal of the esca-associated fungus *Phaeoacremonium aleophilum*. The fungicide properties of ozonated water was firstly assessed *in vitro* by exposing spores to several concentrations of ozonated water (2.2, 4.5, or 13.5 g/m^3 of ozone dissolved into water) and observing the germination rate on plate. Secondarily an *in planta* approach was conducted on grapevine cuttings of cv. Cabernet Sauvignon clone 15. *P. aleophilum* conidia were inoculated in pruning wounds, which then received ozonated (4.5 g/m^3) or sterile water. *P. aleophilum* DNA was quantified by a quantitative polymerase chain reaction (qPCR) 4 and 9 weeks post-inoculation. The effect of ozonated water on plant-defense gene expression was monitored by reverse-transcriptase qPCR (RT qPCR) 48 h post treatment. The results indicate that ozonated water totally suppresses spore germination *in vitro*. In addition, at 9 weeks post-inoculation, fungal development was significantly reduced by 50% *in planta*. RT-qPCR analysis shows that ozonated water did not induce plant-defense-related genes 48 h post treatment. The fungicide properties of ozonated water and the absence of gene induction *in planta* make however ozonated water a promising candidate for limiting grapevine infection by *P. aleophilum* in nurseries.

1. Introduction

Modern agriculture is expected to supply food and protect the environment by using sustainable practices. A major pillar in the strategy of sustainable agriculture is to reduce the pesticide use, which addresses both environmental and social concerns (Tilman et al., 2002). Fungicide inputs are highly important in grapevine production. In vineyards, pesticides have been or are currently applied to prevent the outbreak of vine pests or diseases, to control the surrounding flora, to increase grape yield, and to ensure wine quality (Leroux, 2003; Pezet et al., 2004). A recent review (Compant et al., 2013) indicates that over 30000 ton/year of fungicides or bactericides are currently sprayed on grapevines in France. In Europe, the International Organization of Vines and Wine established that 70000 ton/year of fungicides are used on 3.8 million hectares dedicated to viticulture (http://www.endure-network.eu/). The development of innovative environment-friendly chemicals, for diseases for which no treatment exists, is highly desirable.

Among such diseases are grapevine trunk diseases (GTDs) such as eutypiose, esca, Black Dead Arm (Bertsch et al., 2013) or black-foot disease (Agusti-Brisach and Armengol, 2013), which occurrence has increased dramatically over the last decade and is now one of the major threats for vineyards (Grosman and Doublet, 2012). The comparison of several fungicides and bio-fungicides, such as those containing bio-control agents of the *Trichoderma* genus, as well as treatments or sanitation practices like the hot water treatment, reveals the difficulty of controlling GTDs (Halleen et al., 2007; Rego et al., 2009; Fourie and Halleen, 2004). Other biological candidates for controlling GTDs are bacterial species (Compant et al., 2013). Overcoming the threat of esca and GTDs

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in general, however, will require a finely tuned combination of agricultural practices, sanitation, biological control, and chemical treatments.

Esca *sensus stricto* corresponds to the white rot that develops inside grapevine pith that suffers from a long-term infection (Mugnai et al., 1999). Plants whose trunks are heavily colonized may develop characteristic tiger-striped foliar symptoms of esca without the presence of white rot inside their trunks. Recently, the term "esca *sensus* leaf-stripe disease" has been coined to distinguish these two forms (Andolfi et al., 2011). Foliar symptoms probably originate from toxin transport from colonized wood to the leaves (Andolfi et al., 2011; Spagnolo et al., 2012) and/or from disruption of vessel sap flow (Lecomte et al., 2012). Since sodium arsenite was banned, no alternative treatment has been developed that efficiently controls esca. A recent review suggests that any single treatment cannot efficiently control this complex pathosystem (Bertsch et al., 2013). Several species of fungi have been isolated from the trunks of plants with symptoms of esca *sensus* leaf-stripe disease (Bruez et al., 2011, 2014; Mugnai et al., 1999; Fischer, 2006). *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea* are the main phytopathogens involved in this pathosystem (Bertsch et al., 2013). *P. chlamydospora* and *P. aleophilum* are clearly pathogenic when inoculating *in vitro* grapevine plants (Zanzotto et al., 2008).

P. aleophilum is associated with esca in all wine-producing areas (Mostert et al., 2005) and was isolated on grapevines in vineyards (Rooney-Latham et al., 2005a), in the air (Rooney-Latham et al., 2005b), and in nurseries (Gramaje and Armengol, 2011). The route of infection by *P. aleophilum* is not yet known but infection in nurseries *via* propagation material (Halleen et al., 2003), *via* pruning-wound infections (Eskalen et al., 2007; Aroca and Raposo, 2009), and *via* soil inoculum in vineyards were investigated. Both in-nursery grafting and annual in-field pruning wounds cause significant injuries that could favor the entrance of esca-associated fungi such as *P. aleophilum*. The investigation of compounds that can control or limit the infection of pruning injuries is thus relevant for developing integrated pest management that controls esca.

Ozone appears to be an interesting alternative for controlling fungal pathogens and may be used to control the esca disease. Ozone dissolved in water (called "ozonated water" for convenience) is already used in the agriculture and food industry, notably for controlling post-harvest pathogens (Guzel-Seydim et al., 2004; Park et al., 2008; Miller et al., 2013). For example, dipping table grapes for 1 min in ozonated water decreases gray mold due to *Botrytis cinerea* (Smilanick et al., 2002). In horticulture, ozonated water can clean algae or residual pesticides from water pipes. Irrigating tomatoes with ozonated water increases growth rate (Ohashi-Kaneko et al., 2009), leaf area, and stem thickness, but does not impact stomata conductance, CO_2 assimilation, or fruit production (Graham et al., 2011). In addition, spraying ozonated water on cucumber leaves reduces the development of powdery mildew without causing any visible phytotoxicity (Fujiwara et al., 2009). In fact, no undesired effect has yet been attributed to the use of ozonated water on plants (Fujiwara et al., 2011). Interestingly, the application of ozonated water on *Chrysanthemum morifolium* cuttings promotes the formation and elongation of adventitious roots (Park et al., 2009). Ozone affects microbes directly and/or through free radicals. These compounds oxidize cell walls lipids of microorganisms and may also trigger cell-death signaling. An additional advantage of ozonated water is its low persistence. In fact, ozone quickly decomposes into dioxygen (Staehelin and Hoigne, 1982): its half-life in distilled water is 20–30 min (Khadre et al., 2001). Ozone can decompose also into free radicals with a very short half-life. For example, depending on the temperature, pH, and concentration of free-radical scavengers (Staehelin and Hoigne, 1982), some free radicals decompose within minutes in pure water. This variety of oxidative compounds could explain also the bactericide (Restaino et al., 1995; Young and Setlow, 2004), anti-viral (Wedemeyer et al., 1978), and fungicide (Miller et al., 2013) effect of ozonated water.

To the best of our knowledge, no study has focused yet on the use of ozonated water to control grapevine colonization by fungal agents associated with GTDs. Therefore, the present study investigates the *in vitro* and *in planta* fungicide properties of ozonated water in laboratory conditions. *In vitro* effect of ozonated water was observed with respect to the spore count, shape and germination of *P. aleophilum*. The ability of ozonated water to early reduce the *in planta* colonization of *P. aleophilum* was measured by using molecular tools to quantify fungal DNA. In addition, we used a gene-expression study to investigate the response of grapevine to ozonated water.

2. Materials and methods

2.1. Fungal material

The strain *P. aleophilum* CBS 100398 was used for this study. It was maintained in the dark at 26 °C in a potato-dextrose-agar (PDA) medium (Merks, Germany). Spore suspensions were prepared from four-week-old cultures. A plug of agar colonized by the fungus was placed in a 1.5 mL tube containing 1 mL of de-ionized autoclaved (121 °C, 15 min) water. The tube was vortexed and centrifuged (30 s, 5000 rpm) to precipitate conidia. The plug was removed from the tube. The conidia in suspension were then counted by using a Malassez cell. Only freshly prepared spore suspensions were used for this study.

2.2. Plant material

One-year-old canes of *Vitis vinifera* L. cv. Cabernet Sauvignon clone 15 were harvested in January 2013 in Gaillac (Midi-Pyrénées, France) and stored in the dark at 4 °C after a fungicide treatment consisting of a 1 h bath containing 8-hydroxyquinoline sulfate (0.05% Cryptonol®, Chauvin, France). Canes were then divided into cuttings containing three dormant buds and were cleaned for 30 s in a 20 L water bath containing 10 mL bleach (2.6% active chloride) before being rinsed twice with fresh water. Next, the cuttings were stored overnight at 4 °C in a 0.05% Cryptonol solution. The cuttings were cleaned then three times in baths of tap water and planted in moistened autoclaved glass wool (121 °C; 15 min) contained in plastic trays. Cuttings were watered with autoclaved water (121 °C, 15 min) until budding and rooting. The budded and rooted cuttings were transferred then into pots containing 120 g of soil (1:1:1, v/v, turf, perlite, sand) that had been autoclaved twice (121 °C, 15 min). To avoid reactions to potting stress, newly potted plants were maintained one week in a plant growth chamber (photoperiod 18/6, 45% humidity, 25 °C) before treatment.

2.3. Ozone dissolution in water

Ozone was produced with a LAB2B Ozone Generator (Ozonia, Dubendorf, Switzerland) supplied with pure oxygen. Ozone was dissolved in 10 mL of sterile water by bubbling it through a frit for 10 min. The gas-flow rate was fixed at 10 L/h. The ozone concentration in the inlet gas was 10, 20, or 60 g/m^3. The concentration of ozone dissolved in water was calculated by using Henry's law:

$$[O_3]_l^* = \frac{\rho_{H_2O}}{M_{H_2O}} \times \frac{R \times T}{He} \times [O_3]_g$$

where M_{H_2O} is the molecular weight of water in g/mol, R is the ideal gas constant (0.082 L atm mol^{-1} K^{-1}), T is the temperature in Kelvin, and He is the Henry constant in atm. The Henry constant

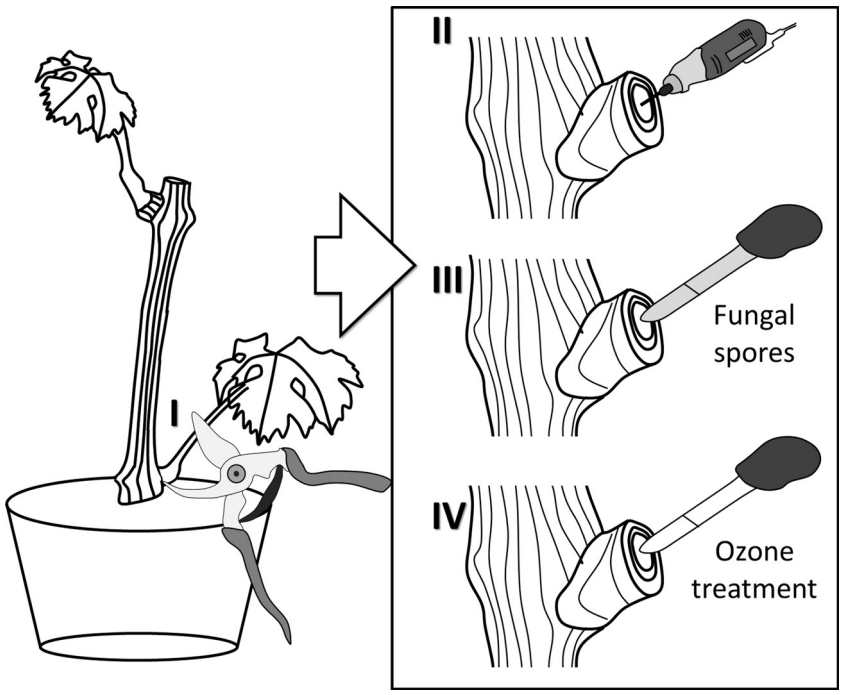

Fig. 1. Pruning-wound model used to study esca disease in laboratory conditions. (I) Pruning lower branch of a grapevine cutting. (II) Drilling injury site deposit solutions of (III) spores of *P. aleophilum*, (IV) ozonated water or, for the control, sterile water.

(He) is obtained by using the Roth and Sullivan (Roth and Sullivan, 1981) equation:

$$He = 3.84 \times 10^7 \times [OH^-]^{0.035} \times \exp\left(\frac{-2428}{T}\right)$$

where ρ_{H_2O} is the density of water density in kg/m^3.

By using this approach, the three different concentrations of ozone dissolved in water were estimated to be about 2.2, 4.5, or 13.5 g/m^3 at 20 °C, pH 7, and 1 atm.

2.4. In vitro *assay of ozonated water treatment on* P. aleophilum *spores*

2.4.1. *Effect of ozonated water treatment on* P. aleophilum *spore germination*

To treat spores with ozonated water, 6 μL of *P. aleophilum* spore suspension (10^7 spores per mL) were transferred into 1.5 mL Eppendorf tubes. Each tube then received 1 mL of ozonated water (2.2, 4.5, or 13.5 g/m^3 of ozone dissolved into water) or autoclaved de-ionized water (121 °C, 15 min) as a control. After 10 min, a volume of 840 μL was pipetted from the surface of each solution and discarded. Fifty microliters were plated on a Petri dish with the PDA medium. Three Petri dishes were prepared for each modality of ozonated water. The twelve plates were incubated then in the dark at 26 °C to compare the germination of spores treated with aqueous ozone with that of spores treated with sterile water.

2.4.2. *Effect of ozonated water treatment on* P. aleophilum *spore count and shape*

To visualize spores within the first hour following ozonation treatment, 100 μL of a 10^7 spores per mL spore suspension of *P. aleophilum* were added to six 1.5 mL tubes. We selected the highest concentration tested in the spore germination study 13.5 g/m^3. Three tubes received 900 μL of ozonated water (13.5 g/m^3) and three tubes received autoclaved de-ionized water (121 °C; 15 min). Spores were quantified at 1 h post treatment (hpt) by using a Malassez cell and observed under a Leica DM750 light microscope (Leica Microsystems GmbH, Germany). Photographs were recorded by using a CMEX-DC5000 camera and its software ImageFocus®v3 (Euromex, Netherlands).

2.5. *In planta assay of ozonated water treatments*

2.5.1. *Effect of ozonated water treatment on* P. aleophilum*–grapevine interactions*

As shown in Fig. 1, three-eyed cuttings (n = 36) were pruned and inoculated with 1×10^5 spores of *P. aleophilum*. Immediately after inoculation, the injury received either 20 μL of ozonated water (4.5 g/m^3, n = 18) or 20 μL of sterile water (n = 18). The samples (N) consisted of three pooled plants that were harvested 4 (N = 6) and 9 (N = 6) weeks post inoculation. Samples were fixed then in liquid nitrogen and stored at −80 °C prior to DNA extraction.

DNA extraction was carried out according to Pouzoulet et al. (2013). Briefly, the samples were lyophilized for 24 h before being ground at room temperature. The powder was incubated for 1 h at 65 °C in a DNA extraction buffer (CTAB 2%, PVPP 2%, Tris base 100 mM, EDTA 20 mM, NaCl 1.4 M, pH 8.0, 5 μL β-mercaptoethanol, 5 μL RNAse A). Half a volume of a chloroform–isoamyl alcohol solution (24:1) was added, and the mixture was incubated on ice. The mixture was centrifuged then and the supernatant was mixed with AP2 buffer from the DNeasy plant mini Kit (Qiagen, USA). The subsequent steps were performed by using buffers, materials, and the protocol supplied with the DNeasy plant mini Kit. The final elution volume was 50 μL, and the samples were stored at −20 °C.

The fungal DNA was quantified according to Pouzoulet et al. (2013), who developed *P. aleophilum* specific primers PalQ (R:5′CGTCATCCAAGATGCCGAATAAAG3′; F*:5′CGGTGGGGTTTTACGTCTACAG3′) targeting a genomic region of the β-tubulin, which is present in a single copy per genome.

2.5.2. *Impact of ozonated water on gene regulation in pruning wound of grapevine cuttings*

We studied whether ozonated water could cause a defensive reaction in plants. Three-eyed cuttings (n = 30) were pruned (Fig. 1,

Table 1
Primer sequences of *Vitis vinifera* L. defense-related genes used in this study.

Gene	Primer sequences	Functions	References
EF1-α	F 5′GAACTGGGTGCTTGATAGGC3′ R 5′AACCAAAATATCCGGAGTAAAAGA3′	Elongation Factor 1 alpha Housekeeping gene	Terrier et al. (2005)
PAL	F 5′TGCTGACTGGTGAAAAGGTG3′ R 5′CGTTCCAAGCACTGAGACAA3′	Phenylalanine ammonia-lyase, marker gene of the salicylic acid pathway and plant response to biotrophs	Aziz et al. (2003) and Thomma et al. (2001)
SOD	F 5′GTGGACCTAATGCAGTGATTGGA3′ R 5′TGCCAGTGGTAAGGCTAAGTTCA3′	Superoxide dismutase Marker gene of plant response to oxidative stress	Spagnolo et al. (2012) and Mittler et al. (2004)
GSTphi	F 5′GTGATTGCCATGCAGAAGAG3′ R 5′TTTTTGGTAGAAACGCTTTATGTT3′	Glutathione-S-transferase phi, marker gene of plant response to oxidative stress	
Lox9	F 5′CCCTTCTTGGCATCTCCCTTA3′ R 5′TGTTGTGTCCAGGGTCCATTC3′	Lipoxygenase 9, marker gene of the jasmonic acid pathway and plant response to necrotrophs	Perazzolli et al. (2010), Thomma et al. (2001), Mittler et al. (2004)
GST	F 5′CACAAAACTACCACCCACCAA3′ R 5′TCCTCACTCTCTTCAATCACTT3′	Glutathione-S-transferase Marker gene of plant response to oxidative stress	
STS8	F 5′AAGACATGTGTTGAGTGAGTATGGTA3′ R 5′CTCGATGGTCAAGCCTGGT3′	Stilbene synthase 8 Antifungal properties	Dai et al. (2012)
PR10a	F 5′GTTTTGACTGACGGCGTTGA3′ R 5′TGGTGTGGTACTTGCTGGTGTT3′	Pathogenesis related protein 10a Antifungal properties	Figueiredo et al. (2008)

panels I and II) and treated with either ozonated water (4.5 g/m^3, $n = 15$) or sterile water ($n = 15$), as depicted in Fig. 1, panel IV. To assess the impact of ozonated water on gene regulation in healthy plants, these plants were not infected. The local injury was harvested then at 48 hpt by using a sterilized secateurs (70% ethanol flamed) and immediately placed in liquid nitrogen and stored at −80 °C for RNA extraction. Wood sections were harvested as close as possible to the injury (0.5 cm deep, 1 cm diameter). One sample consisted of $n = 5$ pooled plants, so the gene induction study was conducted on $N = 3$ samples of ozone-treated plants and $N = 3$ samples made of control plants treated with water.

Samples for RNA extraction were ground in liquid nitrogen by using a Retsch MM300 mortar grinder (60 s, 25 oscillations per second, two cycles; Retsch, Germany) in a 35 mL stainless-steel grinding jar (Retsch, Germany) with 20 mm stainless-steel balls (Retsch, Germany). The subsequent protocol is adapted from Pouzoulet et al. (2013) and Southerton et al. (1998). The powder (100–200 mg) was incubated 10 min at 65 °C in a RNA-extraction buffer (CTAB 2%, PVPP 2%, Tris 300 mM, EDTA 25 mM, NaCl 2 M, pH 8, 2% β-mercaptoethanol) and centrifuged (15 min, 10 000 rpm, 4 °C). The liquid phase was carefully transferred in a new 2 mL tube. One volume of a phenol–chloroform–isoamyl alcohol solution (25:24:1) was added and the samples were then kept on ice as much as possible between the different steps of this protocol. Next, the mixture was centrifuged (30 s, 10 000 rpm, 4 °C) and the supernatant was mixed with 1 volume of chloroform-isoamyl alcohol solution (24:1) and centrifuged. This cleaning step was repeated and the supernatant was transferred into a new tube with 0.5 volume of 8 M LiCl solution. The samples were then stored overnight at −80 °C. The next day the samples were centrifuged (30 min; 10 000 rpm, 4 °C) until a pellet appeared on the bottom of the tube. The pellet was dissolved in 250 μL of SSTE buffer (SDS 0.5%, NaCl 1 M, Tris 10 mM, EDTA 1 mM, pH = 8), mixed with 2 volumes of absolute ethanol and then transferred into a Qiagen cleaning column supplied by the manufacturer (RNeasy plant mini Kit, Qiagen, USA). The subsequent steps were performed by using buffers, materials, and the protocol supplied with the RNeasy plant mini Kit. The final elution volume was 50 μL and the samples were stored at −80 °C.

Early plant response to stress was assessed by measuring mRNA expression of defense-related genes in tissue surrounding the injury. Complementary DNA had to be generated from RNA samples prior to analysis by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). Total RNA was quantified then by measuring the optical density with a Biophotometer Plus (Eppendorf AG, Germany). A DNase reaction (1 U/L 30 min at 37 °C, DNase I, RNase free kit, Fermentas, Canada) was used to ensure that no contaminating genomic DNA was present, and the result was checked by a PCR test that used the total RNA extract as a template and primers of the reference gene Elongation Factor 1 alpha (EF1α, Table 1). The PCR products were then loaded in Tris acetate EDTA-agarose gel (1% agarose, 40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.0). DNA was visualized by ethidium-bromide post staining and illumination by ultraviolet light. The results of RT-PCR were amplified by using a Maxima First strand cDNA synthesis kit for RT-qPCR (Fermentas, Canada), starting from 1 μg of total RNA. qPCR experiments were conducted with an ABI 7500 Real-Time PCR cycler (Applied Biosystems, USA) and the ABI SDS software v.1.4 with the default configuration. The cycling program was as follows: (i) denaturation at 50 °C for 2 min and then 95 °C for 10 min, (ii) 40 cycles of 15 s at 95 °C for denaturation, followed by 1 min at 60 °C for both annealing and extension, and (iii) an additional melting analysis of 40 min from 60 to 95 °C. Eq. (2) $-\Delta\Delta Ct$ was used (Livak and Schmittgen, 2001) to calculate the gene expression relative to the housekeeping gene EF1α. The selected genes were the key gene markers of the defense pathways phenylalanine ammonia-lyase (*PAL*), lipoxygenase 9 (*LOX 9*), pathogenesis-related protein 10a and stilbene synthase 8 (*PR10a* and *STS8*), glutathione-S-transferase phi, glutathione-S-transferase, and superoxide dismutase (*GSTphi*, *GST* and *SOD*). All primer sequences used are indicated in Table 1, which also indicates the metabolic pathways these enzymes are belonging to.

Gene induction was considered biologically significant if the induction factor relative to the reference gene was larger than 2× or

smaller than 0.5×. In both cases there is a two-fold change in gene expression due to the treatment compared to the relative induction observed in non-treated plants. Data from the qPCR analysis were not normally distributed, so the statistical analyses consisted of Kruskal–Wallis rank sum tests done with the software R (R Core Team, 2013).

3. Results

The aim of this study was to test the efficiency of ozonated water to protect grapevine pruning wounds against the esca-associated fungus *P. aleophilum*. To achieve this goal, laboratory tests were done *in vitro* to measure the impact of ozonated water on *P. aleophilum* spores, and *in planta* on its host *Vitis vinifera* L.

3.1. In vitro *fungicide properties of ozonated water*

3.1.1. *Effect of ozonated water treatment on* P. aleophilum *spore germination*

We measured the germination of *P. aleophilum* spores treated with either water or ozonated water. Spores treated with water developed correctly (Fig. 2A). Spores in suspensions treated with the various concentrations (2.2, 4.5, or 13.5 g/m^3) of ozonated water showed no viable germination (Fig. 2B–D). After two months, no development was observed in ozone treated plates.

3.1.2. *Effect of ozonated water treatment on* P. aleophilum *spore count and shape*

By using a Malassez cell, we first counted the number of spores remaining in a suspension treated with ozonated water for comparison with a control sample treated with sterile water. The initial concentration was around 10^6 spores per mL. In both ozone-treated and sterile water-treated suspension, 10^6 spores per mL were found (Fig. 2E). The number of spores in suspension remained relatively constant at the initial number, independent of the treatment received (Kruskal–Wallis chi-squared = 5.7931, df = 2, p-value > 0.05). Moreover we saw that under higher magnification, the spores looked similar (Fig. 2F and G).

3.2. *Effect of ozone on fungal development* in planta

3.2.1. *Effect of ozonated water treatment on* P. aleophilum*–grapevine interactions*

This section investigated if ozonated water applied to an inoculated pruning wound could reduce the development of esca-associated fungal agents. No fungal DNA was detected in non-treated plants (Fig. 3), which confirms that the material used for this study was free from *P. aleophilum*. Upon inoculating with ozonated water (4.5 g/m^3), the difference observed between the ozone treatment and the control at four weeks post inoculation (wpi) was not significant. The number of fungal DNA copies 9 wpi of *P. aleophilum* in the trunk was multiplied by nearly 8×. At 9 wpi, the development of *P. aleophilum* in an inoculated pruning wound was significantly reduced by 50% (N = 6, Kruskal–Wallis chi-squared = 3.8571, df = 1, p-value < 0.05) in plants treated with ozonated water compared with infected injuries treated only with sterile water (Fig. 3).

3.2.2. *Impact of ozonated water on gene regulation in pruning wound of grapevine cuttings*

A set of genes was selected (Table 1) to study the impact of ozonated water on plant metabolism at 48 hpt, with the focus being on genes related to plant response to biotic and abiotic stress (4.5 g/m^3; see Fig. 4). Genes encoding the antioxidant enzymes SOD, GSTphi, and GST were not regulated by our treatments at 48 hpt. Genes related to plant response to biotic stress and coding PAL, PR10a, and STS8 were also not regulated. Only the gene encoding

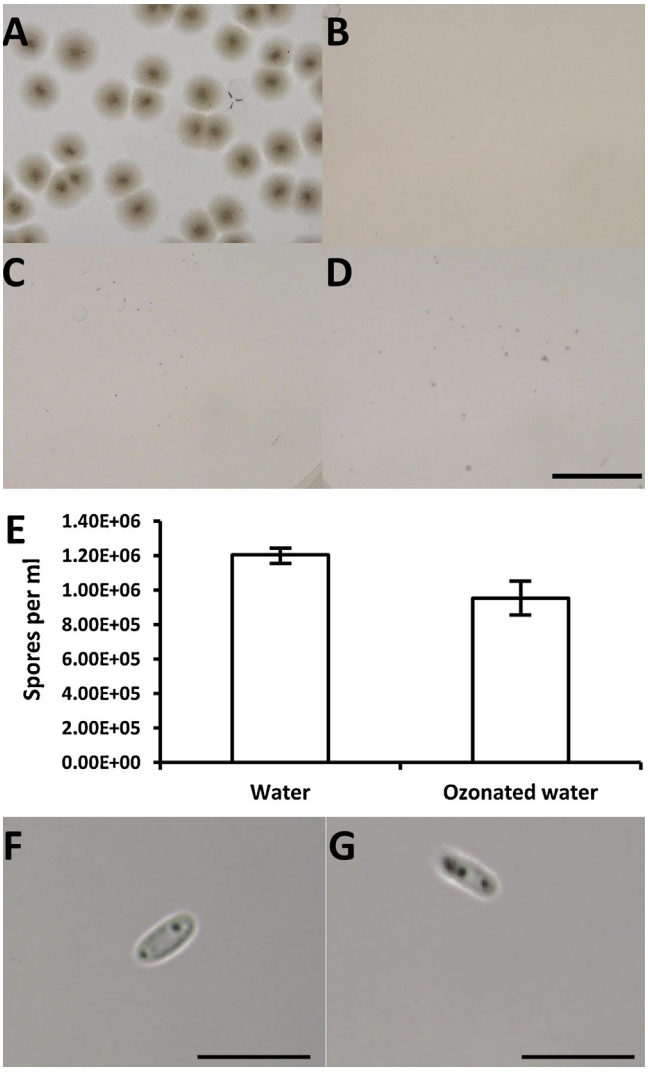

Fig. 2. *In vitro* study of fungicide properties of ozonated water to control *P. aleophilum*. Photographs of young mycelia development of *P. aleophilum* spores treated with (A) sterile water, (B) 2.2 g/m^3, (C) 4.5 g/m^3, and (D) 13.5 g/m^3 of ozonated water (bar = 1 cm). (E) Number of spores per mL in suspension after treatment with water or ozonated water (13.5 g/m^3); photographs presenting similar spore integrity after sterile (F) or ozonated (G) water treatment (bars = 10 μm);.

the protein LOX9 was repressed both in ozone-treated plants and in water-treated plants at 48 hpt compared with undamaged control grapevine cuttings (Fig. 4). This enzyme is involved early in plant responses to wound damage and is a marker of the jasmonic acid pathway (Boubakri et al., 2013b). Overall, this experiment reveals that, at 48 hpt, ozonated water has not served as a natural-defense stimulator for grapevine pruning wounds.

4. Discussion

The solution of ozone in water had a dramatic effect on the *P. aleophilum* spores germination. The experiment *in vitro* presents the fungicidal property of ozonated water on *P. aleophilum* conidia. Firstly the ozone dissolved into water is transformed in dioxygen within 30 min and the solution is not oxidative anymore. Secondly the spores were treated in tubes before being sprayed on Petri dishes. Consequently there was no ozone able to react with organic molecules present in the PDA medium which may form new free radicals. *P. aleophilum* was affected by all ozone concentrations in water, which indicates that this fungal strain is highly sensitive to

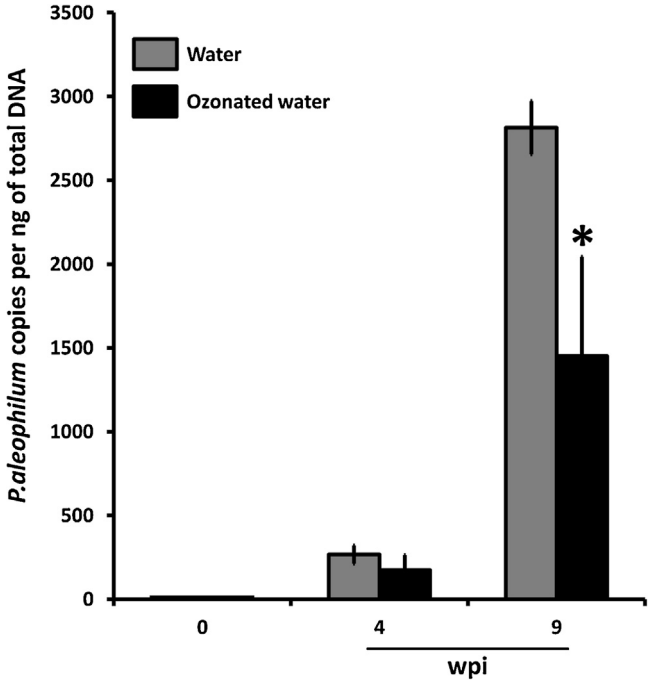

Fig. 3. DNA quantification of *Phaeoacremonium aleophilum* 4 weeks ($N = 6$ samples of three pooled plants) and 9 weeks ($N = 6$ samples of three pooled plants) after treatment. DNA quantification reflects fungal development according to the two treatments: infected pruning wound receiving ozonated water (4.5 g/m^3) 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.".

ozonated water. However, spores were still observable by microscope 1 h after being treated with ozonated water. Thus, the ozone did not directly cause the *P. aleophilum* spores to collapse but could affect the probability of successful spore germination in another way that remains for the moment unclear. Several hypotheses may be suggested to explain this result. First, spore inactivation can be explained by altered membrane integrity. In cell membranes,

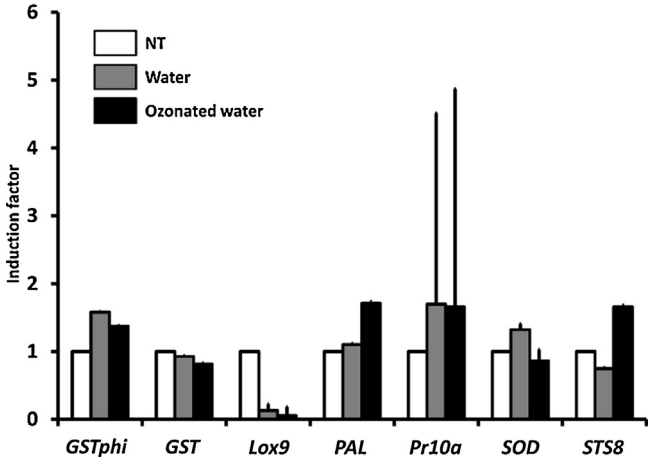

Fig. 4. Relative induction factor of the defense-related genes glutathione-S-transferase phi (*GSTphi*), glutathione-S-transferase (*GST*), lipoxygenase 9 (*LOX9*) phenylalanine ammonia-lyase (*PAL*), *Pr10a*, superoxide dismutase (*SOD*), and stilbene synthase (*STS8*) at 48 h post inoculation by ozonated water (4.5 g/m^3) or sterile water in a pruning wound. Induction factor is relative to the non-treated control plants (induction factor equal to unity) and the housekeeper gene *EF1-α*. Relative inductions are considered biologically significant when >2× and <0.5× compared to the non-treated control.

several families of molecules could be targeted by ozone, such as polyunsaturated fatty acids or chitin, cellulose, or hemi-celluloses, all of which are contributing to the integrity and functionality of the cell wall of fungi (Soriano et al., 2003; Pryor et al., 1995). Ozone, ozonides, and free radicals directly affect spore germination and may induce apoptosis through cell-signaling cascades (Pryor et al., 1995). In the literature, fungal spores are reported to be more resistant than bacteria to ozonated water treatments (Alexopoulos et al., 2013; Restaino et al., 1995). *P. aleophilum* appears to be a suitable model fungus to investigate the mechanisms responsible for the fungicide properties of ozonated water, which is a strong and promising *in vitro* fungicide. Results from this *in vitro* study suggest potential applications of ozonated water as a sanitation agent for vinedressers' materials. This would consist for instance in applying ozonated water on blades, ploughing machine or harvesting machines.

Assuming the desired *in vitro* fungicidal effect of ozonated water is a fact, a successful control tool should target the pathogen at the right stage in its infection cycle. For instance, 5 mM *in vitro* iron sulfate inhibit *P. aleophilum* growth by 40% and appear to be a promising control agent when applied to soil supplements because this compound is absorbed by the roots and redistributed throughout the plant *via* xylem translocation (Fleurat-Lessard et al., 2011).

Although the *in vitro* effect of ozonated water on *P. aleophilum* is dramatic, the *in planta* effect is less drastic. At 9 wpi, a clear decrease of fungal DNA occurs in ozone-treated plants (nearly a factor-of-two decrease). Although ozonated water affects the colonization of a wound by *P. aleophilum*, it is important to note that the pathogen may still infect the grapevine's trunk. This result is an evidence from a laboratory experiment where 10^5 spores suspended in 20 μL were injected into pruning wounds. The wounds then received sterile water or 20 μL of ozonated water, which is a particularly low quantity leading to a low amount of oxidative compounds. In addition oxidative compounds which may react with the fungus is even lower considering the significant quantity of organic compounds in the environment that may potentially react with ozone. Nevertheless those reactions may generate other oxidative compounds susceptible to present a different half life time in the environment. For this reason we cannot exclude that a part of the reduction in fungal development measured *in planta* may come a fungistatic property. However considering the results *in vitro*, we assume that most of the reduction of fungal development observed *in planta* was due to the fungicide property of ozonated water. Finally, this result at 9 wpi remains remarkable because of the very slow growth of *P. aleophilum* in the plant. In fact, β-tubulin DNA copies of *P. aleophilum* between 4 and 9 wpi increased by 8 times, suggesting that around three replication cycles occurred within five weeks in inoculated plants. Protecting pruning wounds with ozonated water is mainly dedicated to grapevine nurseries. A possible application of ozonated water could be to protect wound damage from spore inoculum present on pruning tools, in hydration tanks, or on grafting machines (Aroca et al., 2010). During these steps, plants are either becoming active or are already fully active. Thus, measuring pathogen development in inoculated tissues of young grapevine cuttings by using the tools of molecular biology appears to be an appropriate way to test the fungicidal properties of ozonated water. In this case, quantification reflects the number of initial inoculum that successfully invaded plant tissue *via* a mechanical wounding. Reducing this initial inoculum by 50% is pertinent because an economic study, modeling risk investment in vineyards, showed that reducing the initial inoculum by 25% generates benefits after 12 years compared to conventional practices (Kaplan et al., 2014).

The other potential in-field application of ozonated water may be for protecting pruning wounds. Inoculation in the trunk after

removing a green branch is closer to green pruning than winter pruning. The effect of ozonated water on active tissue was addressed in order to reveal any phytotoxicity or defense elicitation. A set of genes associated with defense in *Vitis vinifera* was selected to investigate whether ozonated water affects the regulation of pathways associated with plant response to abiotic and biotic stresses (Fig. 4). Gene-induction studies of early events in grapevine trunk response are difficult because of the background caused by the wounding damage within the first two days following the treatment. Thus, 48 hpt was a good compromise for this study. Genes encoding the SOD, GST, and GSTphi enzymes are involved in plant response to oxidative stress (Sheehan et al., 2001; Alscher et al., 2002). There was no regulation due to ozonated water or pruning-wound damage in these genes at 48 hpi. In addition, genes encoding PAL, PR10a, and STS8, which are related to grapevine response to biotic stress (Chong et al., 2008; Dai et al., 2012; Boubakri et al., 2013a), were not regulated by ozonated water or pruning-wound damage compared with undamaged plants. Compared with non-treated plants, only the gene *Lox9* responded to both ozonated and sterile water treatments. The lipoxygenase enzyme is associated with the jasmonic acid pathway and to wounding response (Farmer and Ryan, 1992). Thus, this gene could be down regulated at 48 hpt because, compared with the non-treated control plants, the ozone treated plants would be recovering from the intense stress of wounding damage.

Plants treated with ozonated water remained as healthy as the control plants. This observation, together with the study of the expression of defense-associated genes in response to ozonated water suggests a small risk of toxicity due to treatment with ozonated water. The results from Fujiwara et al. (2011) show that repeated spraying of ozonated water does not cause visible symptoms on seedlings of tomato, melon, water melon, eggplant, or cucumber. More research is necessary before envisioning any large-scale application of ozonated water to grapevine culture. Nevertheless, it is encouraging that ozonated water did not yet cause any symptom and did not decrease the yield when applied in the irrigation system of hydroponic tomatoes (Graham et al., 2011). The laboratory model tested in this study was not developed to reflect annual pruning wounds, which occur during the cold season on lignified dormant branches. Further tests are required at lower temperatures on dormant woody tissues to investigate this aspect of protecting winter pruning wounds with ozonated water.

5. Conclusion

This study clarifies the potential of ozonated water to control the esca-associated fungus *P. aleophilum*. It consisted of two major sections: the fungicide properties of ozonated water were observed *in vitro* and *in planta*. This work proposes the first laboratory model based on molecular biology to screen within months for biological or chemical agents for controlling esca-associated fungi. Ozonated water appears to be a strong fungicide candidate for decreasing, but not eradicating, the inoculum of *P. aleophilum* that may infect wounds on cuttings and young grapevine plants in nurseries. Ozonated water should not be considered as a miracle cure for esca, but simply as one tool in the arsenal of tools needed to successfully control esca disease.

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