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MICROBIOLOGY

In vitro inhibitory effect of ozone gas on zoospores and hyphae of *Pythium insidiosum*

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ABSTRACT: This study evaluated the inhibitory effect of ozone gas (O_3) on the mycelia and zoospores of *Pythium insidiosum* and verified the morphological changes it caused on the hyphae. The effects of O_3 were evaluated on the mycelia (n = 21) and zoospores (n = 10) of *P. insidiosum*. O_3 was bubbled into vials with sterile distilled water and mycelium or induction medium and zoospores. After 15 minutes, aliquots of mycelia and zoospores were grown on yeast agar at 37 °C/96 hours. Ozonated flasks were incubated at 37 ºC/72 hours; and every 24 hours, aliquots were subcultured at 37 ºC/96 hours. Transmission (TEM) and scanning (SEM) electron microscopy of O_3-exposed hyphae were performed. The results revealed that O_3 inhibited microorganism growth in all ozonated suspensions. Conversely, in control suspensions, *P. insidiosum* growth occurred within 24 hours of incubation. In the SEM, the treated hyphae presented cavitations and cell wall continuity loss; in the TEM, the organelles and cytoplasmic membrane disappeared, and the cell wall was retracted. We demonstrated, for the first time, that O_3 causes irreversible damage on *P. insidiosum* hyphae and prove *in vitro* oomicidal action on zoospores and hyphae.
Key words: pythiosis, oomycete, oomicidal, ozone therapy, O_3.

Efeito inibitório *in vitro* do gás ozônio sobre zoósporos e hifas de *Pythium insidiosum*

RESUMO: Este estudo avaliou o efeito inibitório do gás ozônio (O_3) sobre o micélio e zoósporos de *Pythium insidiosum* e verificou as alterações morfológicas causadas pela ação do gás nas hifas do oomiceto. O efeito de O_3 foi avaliado sobre o micélio (n = 21) e zoósporos (n = 10) de *P. insidiosum*. O_3 foi borbulhado no interior de frascos contendo água destilada estéril e micélio ou meio de indução e zoósporos. Após 15 minutos, alíquotas de micélio e zoósporos foram cultivadas em ágar levedura a 37 ºC/96 horas. Os frascos ozonizados foram incubados a 37 ºC/72 horas; e a cada 24 horas, alíquotas foram recultivadas a 37 ºC/96 horas. Adicionalmente, realizou-se a microscopia eletrônica de transmissão (MET) e varredura (MEV) das hifas expostas ao gás. Os resultados revelaram que o O_3 inibiu o crescimento do micro-organismo a partir de todas as suspensões ozonizadas. Por outro lado, nas suspensões controle o crescimento de *P. insidiosum* ocorreu em 24 horas de incubação. Na MEV evidenciou-se que as hifas tratadas apresentavam cavitações e perda da continuidade da parede celular; na MET as organelas e a membrana citoplasmática desapareceram e a parede celular estava retraída. Este estudo demonstra, pela primeira vez, que o O_3 causa danos irreversíveis nas hifas de *P. insidiosum* e comprova a ação oomicida *in vitro* do gás sobre zoósporos e hifas.
Palavras-chave: pitiose, oomiceto, oomicida, ozonioterapia, O_3.

INTRODUCTION

Ozone gas (O_3), discovered in the mid-nineteenth century, is a triatomic form of oxygen applied in various areas of veterinary and human medicine (SCIORSCI et al., 2020; CATTEL et al., 2021). The medical use of the gas mixture of O_3–O_2 (95–99.95% oxygen and 0.05–5% ozone), called ozone therapy, involves the dissociation of O_3 in water releasing a reactive form of oxygen, which is a robust cellular oxidant (SCIORSCI et al., 2020). Thus, O_3 has many therapeutic properties and biological activities and can be administered systemically and locally (SCIORSCI et al., 2020; SUMIDA & HAYASHI, 2022). In recent decades, ozone therapy has been widely used in the integrative treatment of various pathological processes and studies on infections caused by bacteria (SONG et al., 2018; LIU et al., 2022), fungi (OUF et al., 2016; VAROL et al., 2022), oomycetes (FERREIRA et al., 2021; CARRIJO et al., 2022; BRAGA et al., 2023), and viruses (CATTEL et al., 2021).

Pythium insidiosum is an important aquatic oomycete and the leading causative agent of pythiosis in mammals, affecting predominantly horses, dogs, and humans. Pythiosis is a life-threatening disease

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with cutaneous, ocular, and systemic manifestations and depending on the clinical form, the available therapeutic options do not always result in its cure (YOLANDA & KRAJAEJUN, 2022).

This eukaryotic organism reproduces asexually in aquatic environments, producing infective zoospores attracted to the tissues of susceptible hosts in these marshy environments (MENDOZA et al., 1996). In the tissues of infected hosts, *P. insidiosum* forms hyphae that are morphologically similar to fungi. However, this oomycete has particularities that distinguish it from microorganisms of the Fungi kingdom, including an incomplete sterol biosynthesis pathway in the cell membrane that makes it intrinsically resistant to most conventional anti-fungal drugs that act on membrane sterols (GAASTRA et al., 2010).

Despite numerous researchers having demonstrated the inhibitory action of different compounds on *P. insidiosum* (PEREIRA et al., 2007; FONSECA et al., 2015; LORETO et al., 2018; VALENTE et al., 2020; IANISKI et al., 2021; STIBBE et al., 2022; IANISKI et al., 2022), it is essential to seek therapeutic options that can be integrated into treatment protocols of the varying clinical forms of pythiosis in animals and humans. In this context, this study evaluated the inhibitory effects of the gas mixture O_3–O_2 on the mycelia and zoospores of *P. insidiosum* and verified the morphological changes caused by O_3 on the oomycete hyphae.

MATERIALS AND METHODS

Tests with P. insidiosum mycelia

The effects of O_3 on *P. insidiosum* mycelia were tested with 21 isolates of the oomycete, including nine standard strains (CBS 1015.55, CBS 575.85, CBS 576.85, and CBS 702.83 from horses, CBS 1194.53, CBS 1194.54, CBS 1194.55, and CBS 673.83 from humans, and CBS77784 from *Culex quinquefasciatus* larvae), 11 clinical isolates from horses, and one from a dog (SISGEN A139392). All clinical isolates were morphologically and molecularly characterized according to AZEVEDO et al. (2012).

P. insidiosum isolates were previously cultured on 0.1% yeast agar and incubated at 37 °C/48 h. Agar blocks from these cultures were then transferred to Sabouraud broth (50 mL) and incubated under agitation (120 rpm) at 37 °C/72 h. Subsequently, the mycelia were drained by filtration and washed with sterile distilled water (dH_2O). A 300-mg aliquot, equivalent to 50–55% transmittance at 530 nm, of each mycelium was transferred to Falcon tubes and suspended in 30 mL of dH_2O. The O_3 concentration was set to 60 μg O_3/mL in an ozone generator (Medplus V, Phlilozon®), fed with pure medical oxygen at a 500 mL/min flow rate.

Ozone gas at 60 μg/mL was bubbled into the flasks for 5 min. After 15 min, fragments of ozonated mycelium were grown on 0.1% yeast agar and incubated at 37 °C/96 h. The O_3-exposed mycelium suspensions were then incubated at 37 °C/24 h, 48 h and 72 h. At each time of incubation, mycelium fragments were removed from the ozonated suspension and subcultured in 0.1% yeast agar at 37 °C/96 h. The cultures were evaluated daily, and the readings were based on hyphae growth every 24 h during five days. The same protocol was used for the control mycelium suspensions (no O_3 exposure), and all assays were performed in duplicate.

Tests with P. insidiosum zoospores

These assays were performed with four clinical isolates (SISGEN A139392), and six standard strains (CBS 1194.53, CBS 1194.54, CBS 1194.55, CBS 673.85, CBS 702.83, and CBS 777.84) of *P. insidiosum*. Zoospores were obtained by zoosporogenesis, as described by IANISKI et al. (2021). This procedure involved transferring culture blocks of *P. insidiosum* on 0.1% yeast agar to Petri dishes containing V8 agar. Sterilized fragments of *Paspalum notatum* were added to the cultures, and the plates were incubated at 37 °C/48 h. Fragments of grass colonized by *P. insidiosum* mycelium were transferred to a Petri dish with 20 mL of induction medium and incubated at 37 °C/24 h under agitation (60 rpm). Afterward, free zoospores in the induction medium were counted in a Neubauer chamber under light microscopy (100X and 400X).

For ozonation, Falcon-type flasks with 30 mL of induction medium containing 10^3–10^4 zoospores/mL were submitted to bubbling with O_3 at 60 μg/mL for 5 min. After 15 min of ozonation, an aliquot (20 μL) of the zoospore suspension was grown on 0.1% yeast agar and incubated at 37 °C/96 h. The zoospore suspensions exposed to O_3 were thereafter incubated at 37 ºC for 24 h, 48 h and 72 h. At each incubation time of the suspensions, 20 μL were subcultured in 0.1% yeast agar at 37 °C and incubated during five days. The cultures were evaluated daily, and the reading was based on hyphae growth every 24 h during 96 h. The same protocol was used for the zoospore control suspensions (no O_3 exposure), and all assays were performed in duplicate.

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed according to the protocol described by VALENTE et al. (2019). O_3-exposed *P. insidiosum* hyphae (CBS 1015.55; 60 μg O_3/mL/5 min) and control hyphae (no O_3 exposure) were fixed in 1 mL glutaraldehyde (2.5%) at 4 °C/48 h. Subsequently, the hyphae were washed in dH_2O and subjected to baths (20 μL/20 s each bath) with increasing ethanol concentrations (30, 50, 70, 95, and 100%), drained, placed on microscope coverslips, and dried in an oven at 37 °C/24 h. Samples were then transferred to stubs, covered with palladium-gold (60 s, 1.8 mM, 2.4 kv), and visualized at 15 kv at 500–1500x.

Transmission electron microscopy (TEM)

P. insidiosum hyphae (CBS 1015.55) were processed according to the protocol of VALENTE et al. (2019) and IANISKI et al. (2021). In summary, the hyphae treated with 60 μg O_3/mL/5 min and hyphae control were fixed in 1 mL glutaraldehyde (2%) and kept at 4 °C overnight, followed by washing twice with dH_2O and thrice with sodium cacodylate (0.1 M), sucrose buffer (0.2 M), and pH 7.4. The samples were then resuspended in osmium and sodium cacodylate buffer (0.4 M, pH 7.4) for 50 min. Afterward, the hyphae pellets were washed with dH_2O and subjected to successive alcohol baths at increasing concentrations of 50, 70 and 90% for 5 min each bath. The samples received three baths with 100% alcohol for 10 min, followed by two washes with acetone for 10 min. The pellets were then submitted to a bath containing 1 mL of acetone and 1 mL of resin solution, and the samples were added to the resin and kept in constant stirring for 1 h. Ultrafine (60 nm) and semi-fine (1 μm) sections were made on a Leica Ultracut UCT using a cacodylate buffer; TEM-generated images were observed on a JEM-1400 microscope (JEOL, Tokyo, Japan).

RESULTS

Ozone gas at 60 μg/mL applied for 5 min on *P. insidiosum* hyphae and zoospores inhibited oomycete growth at all suspensions submitted to ozonization. However, from the control suspensions of hyphae and zoospores, *P. insidiosum* mycelial growth was evidenced in 24 h of incubation at 37 ºC.

The SEM showed that the control hyphae were intact, with regular contours, homogenous surfaces, and turgid aspects (Figure 1A). Nevertheless, the treated hyphae were dehydrated, withered and destroyed, showing various cavitations and loss of cell wall continuity (Figure 1B). The TEM analyses showed that the control hyphae, cell wall, and cytoplasmic membrane were unscathed, and organelles (e.g. mitochondria and Golgi apparatus) were visible inside the cytoplasm (Figures 2A and 2B). In the O_3-exposed hyphae, we observed that the intracytoplasmic organelles disappeared and fine granular material accumulated inside the cytoplasm. The cell wall of these hyphae was retracted, and, in some spots, the cytoplasmic membrane was detached (Figures 2C and 2D).

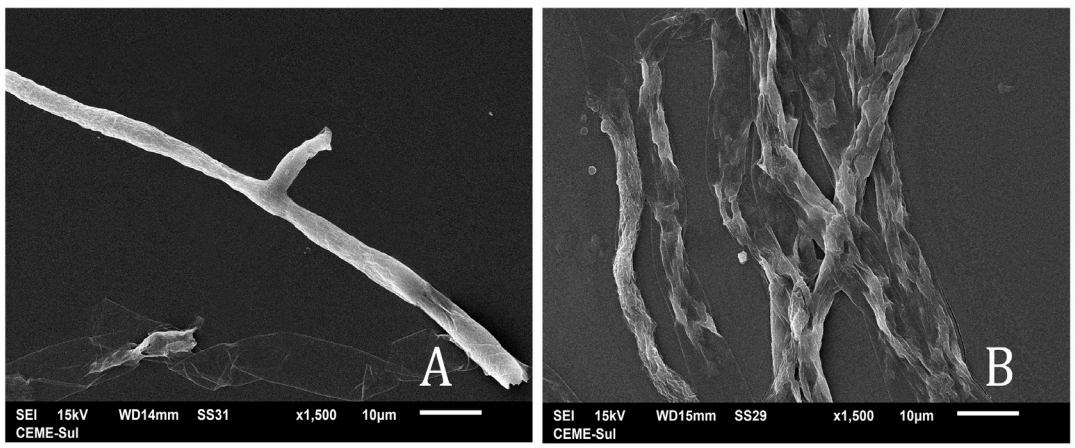

Figure 1 - Ultrastructure of *Pythium insidiosum* (CBS 101555) visualized in scanning electron microscopy (SEM). A) Control sample (no treatment): hyphae of *P. insidiosum* showing regular contours, turgid appearance, and smooth homogeneous surfaces. B) Sample treated with 60 µg O_3/mL. Dehydrated, shriveled, and destroyed hyphae showing multiple cavitation points along the hyphae and cell wall continuity loss.

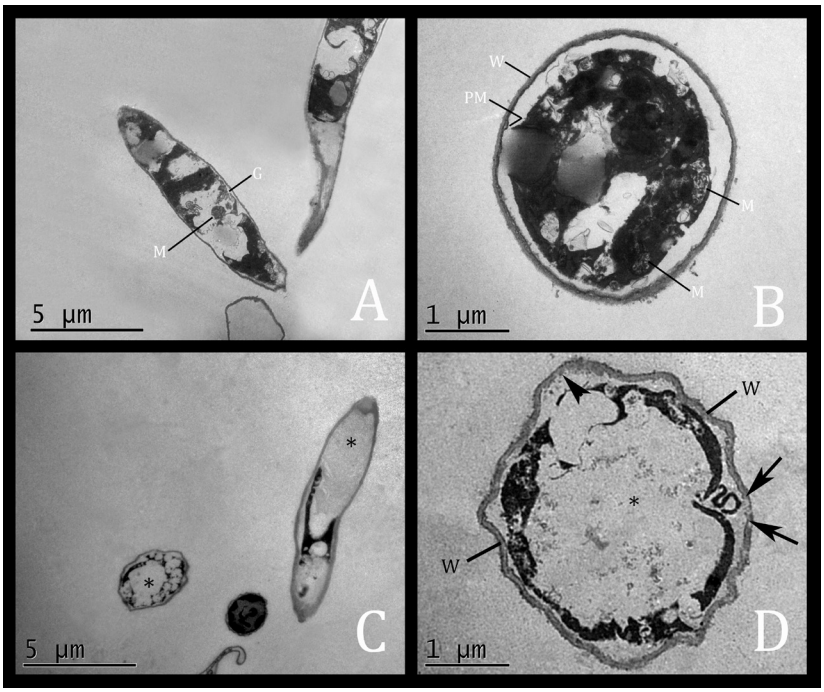

Figure 2 - Ultrastructure of *Pythium insidiosum* (CBS 101555) visualized in transmittance electron microscopy (TEM). A and B) Control sample (no treatment). Hypha showing cytoplasmic organelles such as mitochondria (M) and Golgi apparatus (G), as well as preserved cell wall (W) and plasma membrane (PM). C and D) Sample treated with 60 μg O_3/ mL; C) organelle disappearance and finely granular material accumulation inside the cytoplasm (*). D) Hyphae with retracted cell walls and areas with the missing cytoplasmic membranes (between the arrows and arrowheads).

DISCUSSION

The medical use of the gaseous mixture of O_2–O_3 (95–99.95% oxygen and 0.05–5% ozone), called ozone therapy, has stood out in recent decades as an integrative therapy of various diseases, including skin diseases in animals and humans (ZENG & LU 2018; LIU et al., 2022). Ozone has a broad spectrum of antimicrobial properties, such as action against viruses, bacteria, fungi, yeasts, and protozoa, especially when these agents are in aqueous media (SERRA et al., 2023). Additionally, this study extends the ozone activity spectrum, proving that ozone therapy has a potential activity against *P. insidiosum*, a critical oomycetes pathogen for mammals.

In most affected species, pythiosis is lethal since there is still no standard treatment protocol (VALENTE et al., 2020). Conventional therapies, including drug use, immunotherapy and surgical intervention, are not always fully effective (YOLANDA & KRAJAEJUN, 2022). Thus, given the intricacy of disease and its difficult-to-treat, researchers should develop and include integrative and complementary therapeutic approaches, as ozone therapy, to improve pythiosis treatment and patient outcomes.

In this study, when *P. insidiosum* hyphae and zoospores suspensions were submitted to bubbling with O_3 at 60 μg/mL/5min, we observed *in vitro* growth inhibition of all oomycete isolates. FERREIRA et al. (2021) assessed mycelium exposure of five *P. insidiosum* isolates to O_3 at different concentrations (32, 52 and 72 μg/mL/30 min) in a single application and demonstrated that these treatments did not affect oomycete development. However, when the authors evaluated the exposure of the mycelia to O_3 for three consecutive days, *P. insidiosum* growth was inhibited. In another study, CARRIJO et al. (2022) applied O_3 at 72 μg/mL for 5, 15 and 30 min and at 32 and 52 μg/mL for 30 min on the mycelia of one *P. insidiosum* isolate and observed that all concentrations only inhibited the microorganism growth when applied for 30 min.

The differential of our research, compared to previous studies (FERREIRA et al., 2021; CARRIJO et al., 2022) was the inhibition of growth of *P. insidiosum*

from hyphae (n = 21) and zoospores (n = 10), from clinical isolates and standard strains from different origins, treated with lower gas concentration (60 μg/mL) and shorter exposure time (5 min.). We believed these differences may be due to the method employed and the number and origin of isolates evaluated. In our assays, we attempted to use the method described by FERREIRA et al. (2021) and CARRIJO et al. (2022), although, we detected a leak of O_2–O_3, preventing one from precisely evaluating the gas concentration to which the hyphae were exposed and consequently jeopardizing biosafety due to O_3 toxicity. Therefore, we choose to evaluate the action of O_3 by bubbling using the gas concentration and ozonation time recommended by the Madrid Declaration on Ozone Therapy ($ISCO_3$, 2020). Additionally, ZARGARAN et al. (2017) used the bubbling technique to evaluate the effects of O_3 on *Candida albicans* and verified its effectiveness in inhibiting this yeast.

Ozone is unstable in water and reacts with some components of the aqueous matrix. Nonetheless, ozone decomposes into OH radicals, the most potent oxidants in water. Hence, the oxidation of organic and inorganic compounds during ozonation may occur via ozone, OH radicals, or a combination of both (GUNTEN, 2003; SILVA et al., 2011). These oxidation reactions are likely responsible for the oomicidal activity of the mixture O_2–O_3 reported herein. In addition, through the SEM and TEM analyses, we demonstrated, for the first time, that ozonation causes irreversible morphological changes in *P. insidiosum* hyphae.

OUF et al. (2016) and LIU et al. (2022) suggested that ozone has oxidative effects on eukaryotes of the Fungi kingdom and, upon penetrating the fungal cytoplasm, it disrupts cellular functions and inhibits fungal enzyme production, causing nutrient leakage. ZHANG et al. (2011) employed TEM analyses and observed that O_3-exposed *Pseudomonas aeruginosa* cells showed cytoplasm agglutination, increased cytoplasmic membrane permeability, and irregular cytoplasmic component distribution. The authors also demonstrated the extravasation of potassium, magnesium and adenosine triphosphate by O_3-exposed bacterial cells, proving the increase in cell permeability. It is plausible that ozone caused similar lesions in *P. insidiosum*, since it was observed the loss of intracytoplasmic organelles and fine granular material accumulation inside the cytoplasm of O_3-exposed hyphae. Additionally, changes in the cell wall and cytoplasmic membrane of the hyphae suggest the increase of cell permeability. Despite our promising findings, further research should be conducted to determine the mechanism of action of O_3 on *P. insidiosum*.

CONCLUSION

This study reveals, for the first time, that ozone gas can cause irreversible lesions in *P. insidiosum* hyphae and demonstrated the oomicidal action of O_3 *in vitro* on infective (zoospores) and parasitic (hyphae) forms of this oomycete. Thus, we suggested ozone therapy can be further studied in integrative protocols for treating pythiosis in animals and humans, contributing to finding a cure and improving lesion regression.

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DECLARATION OF CONFLICT OF INTEREST

None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence the content of this work.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, *in vitro* assays, data collection, and analysis were performed by C.Q.B., C.G.Z., A.D.B., H.Z.R, A.M.S., and L.B.I. The SEM and TEM analysis was performed by M.P.S. and D.I.B.P. The first draft of the manuscript was written by C.Q.B, D.I.B.P., and S.A.B. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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