



Electrifying greenhouse agriculture: Cold atmospheric pressure plasma technology for *Pythium ultimum* control

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ABSTRACT

Hydroponic growth of food plants in greenhouses is of rapidly increasing importance to assure future autonomy of food supply, especially in harsher climate zones. Greenhouse culture yields are drastically reduced by pathogenic microorganisms that cause root rot in plants. In Canada, the fungus *Pythium ultimum*, which can survive harsh winter conditions, has a particularly large impact on food production. In this work, we present cold physical plasma treatment of liquids with a gliding arc plasma as a novel approach for combating *pythium* growth in liquid media. This study is based on exploring air or other N₂ + O₂ mixtures as a parameter to identify which plasma treatment is best suited for its anti-fungal activity in different media. If sourced from renewable energy and water, the proposed treatment is intrinsically sustainable. 3 media conditions are explored: first distilled water, to identify the production of highly reactive oxygen species (ROS) and reactive nitrogen species (RNS). Second, an inoculated distilled water is used in conjunction with an ELISA assay as a quick response indicator. Third, a Sabouroud 2 % dextrose broth, is used as a culture media in which oomycetes are grown subsequent to plasma treatment, and hyphal mass is compared between untreated and treated samples. Extracting a subset of 80+ chemical reactions from the available literature and databases, a reaction scheme is proposed accounting for liquid-vapor equilibria (through Henry's coefficients) and reaction rate analysis. The most promising plasma treatment condition was found to be using a 95 % N₂: 5 % O₂ gas mixture with a treatment time of 30 min, reducing hyphal mass growth from 1.8 g to 0.4 g over 1 week in Sabouroud broth. The *pythium* degradation process was observed through scanning electron microscopy (SEM) analysis, showing that Sporangium or oogonia containing structures that terminate the *pythium*'s hyphae have been broken and significantly reduced after plasma treatment.

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1. Introduction

Rising food insecurity due to climate change and inadequate supply-chain control (Aday and Aday, 2020) highlight the need for local and year-round farming technologies such as greenhouse farming. Agriculture in general and indoor farming specifically contribute significantly to greenhouse gases in the atmosphere through the use of carbon intensive nitrogen fertilisers that relies on fossil fuel as cheap source of hydrogen required for its production (Ghavam et al., 2021; Walling and Vaneekhaute, 2020), and through the use of chemically produced pesticides that are also based on fossil fuel educts. As an example, chemically produced fungicides are responsible for between 12 and 30 kg CO₂ emission per kg of produce (Audsley et al., 2009; Pesticide Action Network North America, 2023). Canada's rigorous climate over much of the year strongly favours food production in greenhouses and Canada's agricultural landscape is set to change drastically with the volume of greenhouse crops on track to double by 2025 compared to 2020 (LaPlante et al., 2021). The move towards reliable and climate independent indoor farming, especially in harsher climates such as Canada's, requires decisive steps towards an electrification of greenhouse farming based on renewable sources of energy.

During the humid summer months in Canada, persistent pathogens slow down production in what should be a greenhouse's peak revenue season, resulting in sizable percentages of produce loss. An example of one such destructive pathogen family is the fungus *Pythium ultimum*, which attacks the plants' root system (Van der Plaats-Niterink, 1981; Jarvis, 1992). *Pythium* spp. are plant-damaging, pathogenic oomycetes that are widespread in agricultural soils and water sources. They can inflict significant harm either on their own or in conjunction with other pathogens. *P. ultimum*, in particular, is accountable for various plant ailments such as damping off and wilting in certain crops. While safeguarding against pathogens is essential, relying on chemical fungicides in indoor farming is not sustainable as a long-term solution and may carry potential health risks for agricultural workers and consumers (Van der Plaats-Niterink, 1981; Jarvis, 1992).

In this study, we investigate the use of cold atmospheric pressure plasma as means to replace chemical fungicides with the aim to further the electrification of greenhouse farming. Cold atmospheric pressure plasma sterilization has been intensely studied for agricultural and medical applications over the past few decades (Domonkos et al., 2021; Divya Deepak, 2022). Atmospheric pressure plasma can produce chemically reactive atoms, radicals and molecules, so-called reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Divya Deepak, 2022; Misra et al., 2016; Machala et al., 2012). Reactive oxygen/nitrogen species (RONS) are important for all living organisms and play major roles not only in plasma-based sterilization, but also in fertilization for the growth of plants (Misra et al., 2016; Machala et al., 2012). The correlation between plasma-generated RONS and antibacterial/antifungal effects has been widely discussed (Divya Deepak, 2022; Todorova et al., 2022). Gaseous RONS produced from ambient air by various atmospheric pressure plasma-generating techniques can be transported downstream via the gas flow toward a liquid phase target, for example a stationary or flowing reservoir of tap water (Lu et al., 2016). The resulting plasma treated water (PTW) can be jointly exploited to combat harmful plant-borne microorganisms, as well as provide nitrogen-containing plant nutrients (Todorova et al., 2022). Some of the most frequently investigated RONS are singlet oxygen, hydroxyl radicals, peroxynitrites (HOONO_(aq)/ONOO_(aq)), peroxynitric acid (PNA, HOONO_{2(aq)}), hydroperoxy radicals, ozone (O_{3(g)}/O_{3(aq)}), and hydrogen peroxide (H₂O_{2(aq)}), measured in the gas phase and/or in the liquid phase. The treatment of water by plasma – while requiring initial financial investment in the plasma- and gas-handling infrastructure – is a sustainable technology that is environmentally benign, involving only ambient air, water and electricity.

Plasma disinfection was first studied in the context of surface and tools sterilisation, but it has also extended to food security and industry, where it is used to prevent fungal spoilage. Following the findings on bacterial inactivation and mechanisms of disruption of microorganism functions (e.g. Lerouge et al., 2001; Moisan et al., 2001), and subsequent research in other groups (Niedzwiedz et al., 2019; Ehlbeck et al., 2010; Bourke et al., 2018; Kogelheide et al., 2020; Misra et al., 2019), in his review article, has proposed the following mechanisms for fungal inactivation:

- i) Inhibition of the cell membrane
- ii) Apoptosis of fungal cells
- iii) Morphological changes in the cellular structures
- iv) Loss of permeability and cell leakage
- v) Oxidation of intracellular organelles, fragmentation and release of DNA

The above mechanisms will be compared with the data found in this research.

Contaminated substrates can be treated either directly via the generation of plasma-activated gas (e.g., plasma-processed air, PPA) or indirectly via plasma treated liquids (e.g., plasma-treated water, PTW) (Rathore et al., 2021; Khlyustova et al., 2019; Brisset et al., 2008; Lu et al., 2017). Recent studies demonstrated the inactivation of *P. ultimum* mycelia after treatment with PPA (Wannicke and Brust, 2023). The vegetative state of *P. ultimum* was inactivated and prevention of spore formation was presumed, as no growths of mycelia in any of the plasma treated samples was recorded over a period of 14 days. These PPA experiments highlight the role plasma can play in plant pathogen control. The use of PTW, however, is much more practical and applicable in a greenhouse setting. The technological approach of PTW presented in the present work is based on gliding arc technology (Brisset et al., 2008; Lie et al., 2006; Fridman, 2004; Sampaio et al., 2022). The advantage of gliding arcs is that, due to their transitional nature, they have a high electron density and current comparable to thermal plasmas. However, they operate at a low gas temperature and high electric field, which is more commonly found in non-thermal plasmas. Due to their properties, gliding arc discharges are well-suited for treatment of liquids for pollutant abatement in the scope of sustainable environment (Brisset et al., 2008; Fridman, 2004). To summarize: in this work, we use cold atmospheric pressure plasma to generate fungicidal agents using only electricity, air, and water means to replace chemical fungicides. Our study demonstrates the capability of plasma treated water to combat the plant pathogen *P. ultimum*.

2. Experimental methodologies

2.1. Plasma source

Fig. 1(a, b) shows a schematic representation, and a photograph of the gliding arc device used in this work. The plasma was sustained from a PVM-500 resonant high voltage power supply with a 2500 secondary coil (Information Unlimited Inc., Mont Vernon, NH, USA). The resonance frequency was around 24.5 kHz, the voltage was kept at 60 % of the single voltage mode, typically 4–6 kV across the electrodes, and the current measured on the power supply was 0.3–0.4 A, for an average power of 27 W. One of the two gliding arc electrodes was connected to the power supply, while the other one was grounded. The system was not cooled otherwise than by the air flow and the initial temperature of the water (both at room temperature).

The electrode geometry facilitates the basic properties of a gliding arc: initiated as a thermal arc with gas temperatures around 3000–5000 K, the moving gas flow and expanding electrode gap distance transforms this initially thermal arc into a non-thermal plasma, cooling the temperature to below 1000 K. The electron temperature is about 1 eV and the vibrational temperature remains elevated (Fridman, 2004). About 75–80 % of the electric energy can be dissipated in the non-thermal plasma region. The hybrid thermal and non-thermal character of a gliding arc make it an ideal plasma source for environmental applications and gas conversion.

In the gas phase, atmospheric pressure cold plasmas in air (or other $N_2 + O_2$ mixtures) contain charged particles (electrons and ions) and reactive species such as ozone (O_3), singlet oxygen ($^1O_2$), and several radicals, mainly superoxide (O_2^-), hydroxyl ($^{\bullet}OH$), nitric oxide (NO), and nitrogen dioxide (NO_2). The dissolution of these species in the adjacent water bath (see Fig. 1(a)) then gives rise to a diversity of short-lived species (μs to second lifetime) and longer-lived species (second to hours/days lifetime) species, cf. to (Tampieri et al., 2023). Of particular importance for biological effects, there are three primary, long-lived species: hydrogen peroxide (H_2O_2), nitrite (NO_2^-) and nitrate (NO_3^-) (Brisset et al., 2008; Machala et al., 2019). Consequently, quantitative detection of these three species will represent the basic criterion for plasma source characterization, and effectiveness with regard to the biological activity associated with PTW. This is described in some detail in Section 2.3. A key variable examined in this work was the N_2 / O_2 gas mixture ratio R :

$$R = [N_2 / (N_2 + O_2)] \quad (1)$$

R was varied from the subset including (0 0.2 0.4 0.6 0.8 0.9 0.95 1). A second key variable was duration of plasma exposure of the water bath (10 and 20 min for ELISA; 15 and 30 min for hyphal mass growth, both described in 2.4), the aim being to optimize treatment effectiveness, while maintaining other conditions nominally constant, for example gas flow rate (5 slm total) and applied power.

In order to determine the reaction rates at each gas mixture, tap water underwent plasma treatment to identify the species generation over time (at 0, 0.5, 1, 2, 3 and 4 min) for each of the ratios R identified above, and in technical triplicates. The characteristics of the tap water are recorded for the municipality of Montreal (Rémillard et al., 2023).

From these first points, a linear regression is obtained and allows to quantify the reaction rates of the longer-lived species as a function of time. This production is subject to change as a function of pH and temperature; it is only valid if, as in our case, the total concentration of each longer-lived species is below its respective solubility limit. Performing the experiments with dextrose broth directly, instead of with tap water, was prohibited due to the natural optical absorption of dextrose, both in the 407 and 540 nm regions, which interferes with interpretation of the colorimetry results.

2.2. Design of experiment

In this manuscript, the investigation was first carried out to identify the 3 major reactive species generation (NO_2^- , NO_3^- and H_2O_2) while varying the ratio of nitrogen to oxygen (R). This was done by sampling $3 \times 100 \mu L$ (in triplicates for each reactive specie) from 100 mL tap water at intervals of 0, 0.5, 1, 2, 3 and 4 min subsequent to plasma exposure.

In order to identify the efficiency of the plasma treatment with regards to pythium inactivation, the second test was to use the ELISA test for 8 conditions: (Inoculated and distilled water, $R = 0$: 10 and 20 min, $R = 0.8$: 20 min, $R = 0.95$: 10 and 20 min, and $R = 1$:

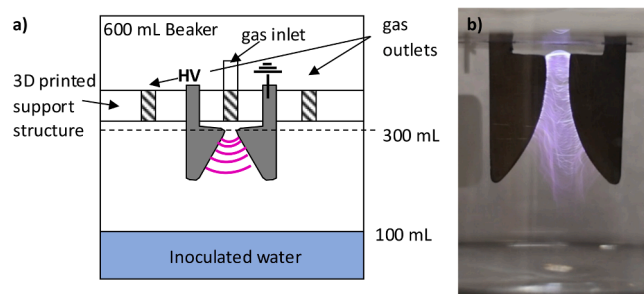


Fig. 1. (a) schematic representation; (b) photograph of the gliding arc device used in this work. The total gas flow is solely composed of nitrogen or oxygen in this exploration, with a total flow of 5 slm. Two holes are added to the cover structure (ca. 5 mm $\varnothing$), and they serve as gas outlets, as such, the reactor is open, but the flow is largely constricted within the treatment chamber.

20 min). The samples were derived from inoculating distilled water with two pythium agar plates in 700 mL: 6 x 100 mL were used for the plasma treatment conditions, while the extra 100 mL was used for the inoculated water condition. The samples were filtered and concentrated; 3 technical replicates were collected before following the ELISA procedure.

As a second complementary method to the ELISA, a hyphal mass growth was conducted for 7 conditions: a control sample, $R = 0$: 15 and 30 min, $R = 0.8$: 15 and 30 min and $R = 0.95$: 15 and 30 min. The initial sample consisted of 10 mL of SBD containing a highly concentrated *Pythium ultimum* content inoculated in 90 mL of distilled water. The sample then underwent plasma treatment, from which a subsequent 3×10 mL were divided and added to 240 mL of SBD for a 7-day growth cycle.

Finally in order to understand more about the process of *Pythium* inactivation and underlying chemistry, 2 samples (control and $R = 0.95$, 30 min) were taken for SEM imaging, and the pH and Temperature were investigated for both tap water and SBD conditions.

The conditions are summarized in Table 1 below. Further details about each process are presented in the subsequent sections of the methodology.

2.3. Materials and chemicals

N_2 gas and O_2 gas (99.9 + % purity, alphagaz 1) were acquired from Air Liquide Canada, Ltd., Montréal. Sabouroud 2 % dextrose broth (SDB), corn meal agar (CMA), phosphoric acid H_3PO_4 (10 % v/v), stabilized reagent grade hydrogen peroxide H_2O_2 (3 % w/w), sulfuric acid H_2SO_4 (4.00 ± 0.04 N, 2 M) and 96-well plates (Thermofischer BioLite™ #130188) were obtained from Fisher Scientific (New Hampshire, USA). N-(1-naphthyl) ethylenediamine dihydrochloride (≥ 98 %), titanium(IV) oxysulfate (anhydride); sodium azide NaN_3 (≥ 99.5 %), sodium nitrate $NaNO_3$ (≥ 99.0 %), sodium nitrite $NaNO_2$ (≥ 97 %), chloroform $CHCl_3$ (≥ 99.5 %), were obtained from Sigma-Aldrich. Nitrate reductase enzyme preparation (#780010), nitrate reductase cofactor preparation (#780012) and nitrate/nitrite assay buffer (#780022) were distributed by Cedarlane Labs for Cayman Chemicals. The *Pythium* spp. ELISA kit was purchased from Creative Diagnostics (New York, USA). Pure *P. ultimum* was provided by the Research and Development Institute for the Agri-environment (IRDA, Québec, Canada).

2.4. Detection of generated reactive species in PTW

Deionized (DI) water (MilliQ, 18 M Ω m) was used when dilution was required. Furthermore, the following liquid diagnostics methods were evaluated in a 96-well plate, using a TECAN Infinite M Nano⁺ plate reader in single absorbance mode ± 4 nm, with 25 flashes, and 2 repetitions.

2.4.1. Determination of nitrite (NO_2) concentration

NO_2 concentration was estimated with an in-house prepared Griess assay and calibration solution, as described in (Clesceri et al., 1999), with reagent type A being composed of N-(1-naphthyl)ethylenediamine dihydrochloride in water at 0.0039 mol/L. Reagent B of the standard Griess assay was obtained by combining sulfanilamide (0.0581 mol/L) in phosphoric acid (5 % w/w; diluted from 10 %). The final Griess reagent was formed by adding reagents A and B together in a 1:1 ratio. 100 μ L samples of water are then pipetted (in triplicate) into a 96-well plate and each combined with 100 μ L of the Griess reagent, to form a purple azo dye after 15 min. These are then measured by absorption at 540 nm with the Tecan plate reader. The detection limits of this method were determined with dilute solutions of $NaNO_2$ stabilised with $CHCl_3$ in the [3–300 μ M] range. Higher concentrations (>300 μ M) can be measured by dilution in DI water or in nitrate buffer, albeit with less accuracy.

2.4.2. Determination of nitrate (NO_3) concentration

NO_3 concentration was determined with a commercial kit from Cayman Chemicals, prepared according to the supplier's instructions. Briefly, a buffer assay was prepared (100 mL DI water employed for its preparation); 1.2 mL of the buffer was used to reconstitute the enzyme and cofactor, respectively. First, 80 μ L of the target liquid is sampled, to which 10 μ L of the enzyme preparation was added, as well as 10 μ L of the co-factor, yielding a 100 μ L sample (repeated in triplicate). The enzyme was left to react for

Table 1
Analysis, conditions, and sample size summary.

Analysis type	Conditions	Replicas	Total samples
Reactive species	$R = [0, 0.2, 0.4, 0.6, 0.8, 0.9, 0.95, 1]$: 0, 0.5, 1, 2, 3 and 4 min	Technical: 3	$9 \cdot 6 \cdot 3 \cdot 3 = 486$
ELISA	Negative and positive control Inoculated and distilled water.	Technical: 3	$10 \cdot 3 = 30$
Hyphal mass growth	$R = 0$: 10 and 20 min, $R = 0.8$: 20 min, $R = 0.95$: 10 and 20 min, and $R = 1$: 20 min. Control	Technical: 3	$7 \cdot 3 = 21$
pH and Temperature	$R = 0$: 15 and 30 min $R = 0.8$: 15 and 30 min $R = 0.95$: 15 and 30 min $R = 0$: 30 min, distilled water and SBD $R = 0.8$: 30 min, distilled water and SBD $R = 0.95$: 30 min, distilled water and SBD		6
SEM	Control, $R = 0.95$: 30 min		2

1 h at room temperature, to reduce the NO_3^- to NO_2^- , before adding the 100 μL of our previously prepared Griess reagent. Second, 100 μL of the target liquid was immediately treated with the Griess reagent, as described in section 2.3.1. The total NO_3^- concentration was found by subtracting the amount of NO_2^- from the initial sample to that of the reduced nitrite after 1 hour. Calibration with NaNO_3 dilutions demonstrated that this method is valid for [3–200 μM].

2.4.3. Determination of hydrogen peroxide (H_2O_2) concentration

H_2O_2 concentration measurements serve as a useful indicator of ROS, in general. It was measured here as described in (Tarabová et al., 2018), with a few modifications, so as to increase the absorbance and lower the detection limit. Titanium oxysulfate was diluted to 0.1544 mol/L in 2 M sulfuric acid, and briefly heated and agitated to facilitate the dilution, as it is near the solubility limit. A sodium azide solution was prepared by dilution to 60 mM. This second solution's role is to inhibit reaction between H_2O_2 and NO_2^- , and thus needs to be added immediately after sampling. A triplicate sample of 200 μL was pipetted, to which 20 μL of NaN_3 solution and 100 μL of titanium oxysulfate solution was added to the 96-well plate cell. Calibration with stabilized H_2O_2 solution demonstrated the validity of this measurement over concentrations ranging from 10 to 500 μM .

2.4.4. pH and temperature measurements

Before the chemical characterization of samples, water and SDB samples were tempered for about 20–30 minutes to reach room temperature (21 °C) and the pH was determined using an APERA LabSen 214–6 probe. The pH and temperature were also measured after 30 min of plasma treatment using $R = 0, 0.8$ and 0.95 in correlation to the hyphal biomass experiments.

2.5. Quantitative determination of *Pythium* inactivation

2.5.1. Semi-quantitative determination of *Pythium* spp. with an enzyme linked immunosorbent assay (ELISA) test

Water samples were filtered using a 0.45 μm nitrocellulose membrane following PTW exposure, to recover microorganisms on a small surface area. Within 30 minutes, the entire sample volume was filtered. Membranes were then placed in 1.5 mL tubes containing the kit's conjugate/sample buffer and vortexed for 60 sec to create a solid-liquid extraction sample. A sterile clamp was used to withdraw the membrane from the fluid. *Pythium* spp. concentrations in the extracted samples were determined using an ELISA kit (Creative Diagnostics, New York, USA). The plate was photometrically read at 405 nm, and the findings were compared to the controls. The ELISA kit's negative and positive controls were employed (Schroeder et al., 2013).

A concentrated solution containing two agar plates of *Pythium ultimum* was prepared and diluted 1:100. This solution was then used to inoculate four distilled water samples for plasma treatment. The plasma treatment consisted of a discharge in different gas ratios ($R = 0, 0.8, 0.95$), with exposures lasting 10 and 20 minutes. The analyses were carried out in technical triplicates on 96-well plates, i.e. three wells per sample. The ELISA kit included a negative and positive controls for *Pythium* spp. A blank control (non-inoculated distilled water) as well as a matrix control (inoculated distilled water) were also added to the ELISA plate to validate the method and compare the results before and after plasma treatment.

2.5.2. Hyphal biomass harvesting

Pythium are usually characterized by their production of coenocytic hyphae (singular: hypha), long, branching, filamentous structures. In most fungi, hyphae are the main mode of vegetative growth, and are collectively called a mycelium. A hypha consists of one or more cells surrounded by a tubular cell wall (Van der Plaats-Niterink, 1981; Jarvis, 1992; Sutton et al., 2006). Hyphal mass growth was conducted after the plasma treatment as a *Pythium ultimum* viability indicator.

Pythium ultimum isolated from plants was cultured in corn meal agar (CMA) at 25 °C for 24 h. When the strains were ready for testing, 5.0 mL of 2 % Sabouraud dextrose broth (SDB) was added to each flask and statically incubated at 25 °C for 72 h. Following incubation, aliquots of the hyphal mass (10 mL) from each flask was removed and subcultured into 250 mL flasks containing 100 mL SDB (Vilela et al., 2015). Strains were cultured at 25 °C for two days on a shaker incubator at 160 rpm. A fungal suspension (10 %) was mixed with distilled water, which was then plasma treated. Following this, 10 mL of each treated sample was placed into a 250 mL flask containing 100 mL SDB and incubated for 7 days at 25 °C. By the end of the incubation period, each of the hyphal biomasses were harvested by centrifugation at 5000 rpm for 15 min. The collected hyphae were oven-dried in air at 60 °C for 2 h, then weighed (Vilela et al., 2015).

2.6. Scanning electron microscopy (SEM)

To assess the effect of plasma treatment, we conducted SEM analyses. Following a given plasma treatment, the hyphal biomass samples were preserved in Karnovsky's fixative solution (2 % v/v para-formaldehyde and 2 % v/v glutaraldehyde in 1x PBS) overnight. Samples then underwent post-fixation using 2 % osmium tetroxide, followed by dehydration using a series of ethanol solutions of increasing concentration (from 50 % to 100 %). Finally, ethanol was substituted with hexamethyldisilazane (HMDS), and the samples were coated with a thin layer of carbon (Quorum, Q150T plus, UK) (Guo et al., 2022). Analyses were performed on a Hitachi TM3030 plus SEM (Hitachi High-Tech Manufacturing & Service Corp, Japan).

2.7. Statistical analysis

To examine the significant changes between trials, a student *t*-test ($P < 0.05$) was used to compare the inoculated water to that of

the plasma treated samples for the ELISA test, while a one-way ANOVA with the Tukey test ($P < 0.05$) was utilized for the hyphal biomass evaluation. The findings of three replicate trials were provided as the mean and standard deviation. For data interpretation, Prism 7 (GraphPad, Inc.) was used.

3. Results and discussion

3.1. Liquid phase analyses

Fig. 2 shows a plot of measured NO_2^- and NO_3^- concentrations as a function of R , the plasma gas composition. Peroxide measurements fell below the detection limit ($5 \mu\text{M}$) for all investigated conditions.

The plasma generated NO_x molecules are long lived species and are indicators of the successive phases of chemical interactions. A more detailed explanation of the NO_2^- and NO_3^- production will be given in Section 3.4, showing the most probable reaction pathways.

The three selected conditions for the successive treatments, namely $R = 0$, $R = 0.8$ and $R = 0.95$, produce distinct amounts of the long-lived species. $R = 0$ is chosen as the most oxidative composition. $R = 0.8$ is the closest representative to air. $R = 0.95$ is known to create highly reactive nitrogen species. The first produces very little NO_2^- or NO_3^- ; the second produces $13.6 \pm 0.9 \mu\text{M}/\text{min}$ of NO_2^- , and $2.1 \pm 0.5 \mu\text{M}/\text{min}$ of NO_3^- . The third condition, with the most atomic nitrogen produces $11.6 \pm 0.1 \mu\text{M}/\text{min}$ of NO_2^- , and $1.6 \pm 0.2 \mu\text{M}/\text{min}$ of NO_3^- .

The pH varies differently for tap water and for SDB, due to different buffering capacities. The starting pH value for tap water is around pH 7 and the pH of SDB is normally around $\text{pH } 5.6 \pm 0.2$. Plasma treatment of tap water for 30 minutes leads to a decrease of pH from neutral to 3.20 for $R = 0.8$ and 3.10 for $R = 0.95$. In the SDB, the same conditions attain a pH of 4.56 and 4.53 respectively. Lowering pH was shown to reduce initial growth of *Pythium* in soil (Bolton, 2009; M. Alhussa, 2012). The pH value of 4.5 in SDB does have a mild impact in the initial growth of *Pythium ultimum*, but this is not considered to be the dominant factor for stunted growth. A water pH value of 4 or below would have a bigger impact on *Pythium* growth but would have a detrimental effect on the plants.

The plasma treatment leads to an increase in temperature of the liquid over time. The highest temperature was 37.1°C (after 30 minutes of plasma treatment) from the initial 20°C , for a 100 mL sample. This temperature increase corresponds to about 4 W thermal power dissipated into heating the liquid phase (out of the ~ 27 W dissipated by the plasma). The measured temperature increase is excluded as a cause for *P. ultimum* inactivation, as described by Döring et al.: *P. ultimum* exhibits the ability to endure temperatures up to 40°C and drought (-50% of moisture content, w/w) for a period of 4 days (96 h) (Döring et al., 2020).

3.2. *Pythium* spp. quantification by ELISA and hyphal biomass measurements

Fig. 3 shows that the inoculated water has a higher absorbance than that of the filtered distilled water, the latter having a similar detection threshold to that of the negative control of the ELISA kit. For the $R = 0$, 10 min, $R = 0.8$, 20 min, and $R = 1$, 20 min treatments, the results are comparable to that of the initial sample, suggesting no decontamination. The only sample that exhibited a significant difference with the inoculated plasma treated water samples is $R = 0.95$, 20 min treatment, which identifies it as the optimal condition for *pythium* inactivation by plasma. As most of the inactivation is expected at a low O_2 admixture, only the longer treatment times were considered for $R = 0.8$ and $R = 1$ to verify that the maximal inactivation was indeed at $R = 0.95$. The tests using

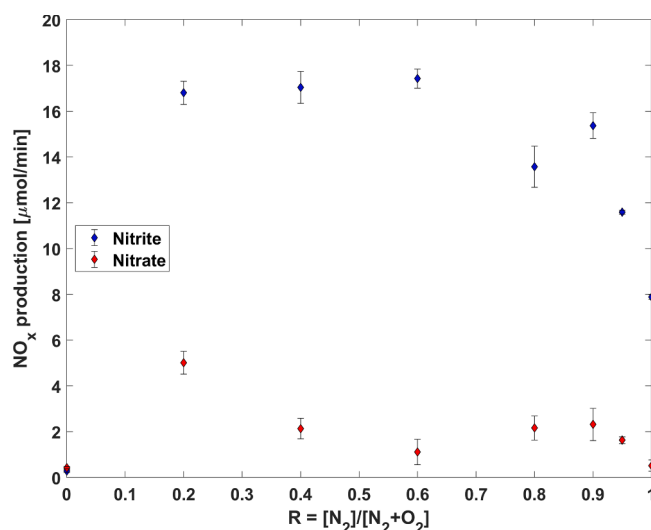


Fig. 2. Analytical results for production of nitrite NO_2^- and nitrate NO_3^- , as a function of plasma gas composition R . Obtained from a linear regression of $n = 6$ samples.

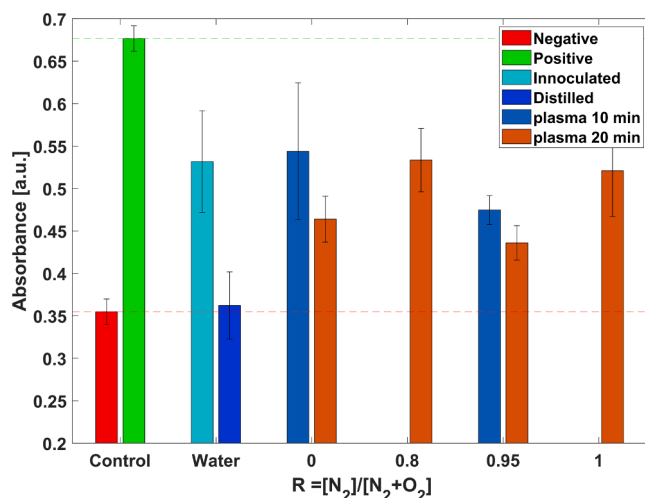


Fig. 3. ELISA results for various *Pythium* spp. samples. The *Pythium*-inoculated distilled water is at the same concentration as samples which undergo plasma treatment. The PTW was as follows: $R = 0$: 20 min, $R = 0.8$: 20 minutes, $R = 0.95$: 10 and 20 minutes, and $R = 1$: 20 min. The error bar corresponds to 1 standard deviation while the bars represent the average of the technical triplicates.

the hyphal mass growth therefore aimed at longer treatment times (15 and 30 min).

To determine the influence of plasma treatment on hyphal biomass, different hyphal biomass was observed in the growth flasks after 7 days of static incubation (see Fig. 4a). The hyphal biomass was collected, dried and weighed to assess the impact of plasma treatment on the hyphal growth. The determined hyphal weights exhibited a significant reduction in case of $R = 0.95$ plasma treatment for 30 min, with weights of 0.38 ± 0.03 g in comparison with control weight of 1.83 ± 0.04 g. The results of $R = 0.95$ treatments for 30 and 15 min are compared in Fig. 4b, where the former is seen to be much smaller. This result is expected, as the longer time of treatment increases the production of RONS that can damage the cell membranes, proteins, and DNA of the microorganisms, leading to their inactivation and death (Xu et al., 2021). On the contrary, the observation of higher hyphal biomass when $R = 0$ was utilized raises intriguing questions about the potential role of ROS in fostering fungal growth (Zhou et al., 2019). Fungi, like many microorganisms, rely on oxygen for cellular respiration, a vital process that generates energy for their metabolic activities. High oxygen levels in the growth medium enhance respiration, allowing the fungi to metabolize nutrients more rapidly and efficiently. This results in accelerated fungal growth rates and can often lead to larger, more robust fungal colonies (Braissant et al., 2020).

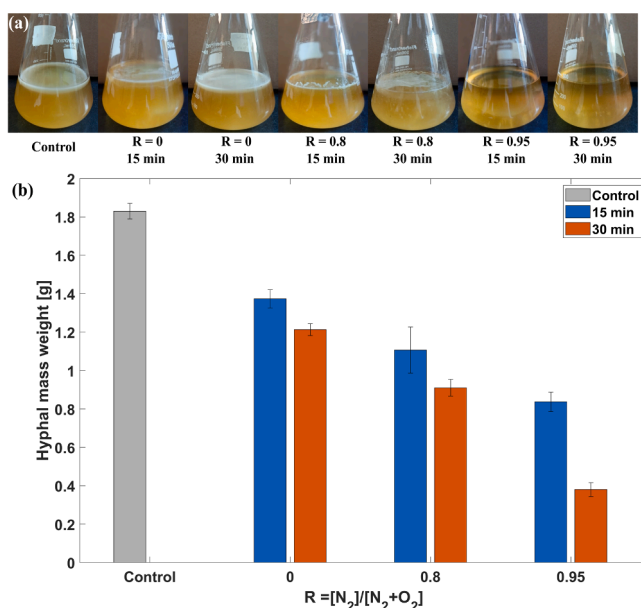


Fig. 4. Effects of different plasma treatments on the growth of hyphal biomass (a) and the variation of the biomass weight after 7 days of static incubation at 25 °C (b). The error bar corresponds to 1 standard deviation while the bars represent the average of the technical triplicates.

3.3. SEM analyses of hyphal biomass

SEM analysis of the control sample group (depicted in Fig. 5a) revealed a substantial presence of hyphal biomass. The hyphal structures displayed characteristic linear patterns with typical thicknesses in the range of $\sim 10\ \mu\text{m}$, concurrent with observations by (Van der Plaats-Niterink, 1981). Additionally, the zoosporangia or oogonia attached to the ends of the hyphae filaments remained intact, with an average size of about $15\ \mu\text{m}$. In Fig. 5b, we can observe alterations in hyphal biomass following a 30-minute $R = 0.95$ plasma treatment. The PTW treatment is seen to have resulted in changes to the mycelial structure, folding of hyphae and a notable reduction in hyphal mass; furthermore, the hyphae filaments appear to have lost some of their characteristic branching. Additionally, the ends of the hyphae filaments, normally containing spores, showed a significant germination reduction, indicating a suppression of their activity due to the treatment. The SEM images also displayed substantial deformations and collapse in hyphal walls and changes in surface texture. In addition, most of the hyphae became thinner and twisted, with typical size ranging from 6 to $8.5\ \mu\text{m}$. The treated samples showed broken hyphae, with noticeable collapses and compressions in hyphal structures. These transformations induced by the PTW treatment may likely be attributed to damaged cell envelopes due to etching of macromolecules (Recek et al., 2018): PTW exposure thereby likely results in damage to cell walls, rendering them permeable and facilitating the release of intracellular components. Altogether, these combined effects ultimately cause collapse and compression of the *P. ultimum* mycelia, rendering them inactive (Misra et al., 2019).

3.4. Overall discussion

Because of the N_2 molecule's very high bond strength, $958.4\ \text{kJ}\cdot\text{mol}^{-1}$ ($\sim 10\ \text{eV}$), it is chemically quite inert. Using a combination of both thermal and non-thermal processes, it is possible to convert N_2 into NO and successively to NO_2 and HNO_3 through the Zeldovich process (Fridman, 2008). Using the reactions proposed by (Machala et al., 2019; Lukes et al., 2014; van Gils et al., 2013) and completing with those found on the NIST database, we propose the chemical model presented at Fig. 6. Starting from the major species produced in the plasma phase, these interact with the species present in the gas phase by collision and diffusion. The species transport to the liquid phase is then controlled by species-specific Henry's law solubility coefficients. Finally a chemical pathway of the liquid species is proposed in accordance to the species which can be readily measured, such as nitrites, nitrates and H^+ ions. In both the gas phase and the liquid phase, the thickness of the arrows indicates the order of magnitude behind the reaction rate enabling for a visualization of the most dominant processes.

The chemical pathways shown in Fig. 6 help to explain the chemical results in Fig. 2, which demonstrated the formation of both NO_2^- and NO_3^- , as the R ratio increases. Regarding the liquid reactions, Fig. 6 shows mainly the nitrogen related species in detail rather than the oxygen dominated species, because species that lead to H_2O_2 are not dominantly produced in the glide arc plasma. The liquid phase chemistry is predominantly defined by the initial gas chemistry and resulting gas composition. The following processes are most dominant for each R -value:

- At $R = 0$ (100 % O_2), the production of both NO_2^- and NO_3^- is almost null. The low production is associated to the traces of nitrogen present to be converted into NO_x .
- At $R = 0.2$, the highest production of NO_3^- is observed. As O_2 is in ample supply, it increases the production of N_2O , O_3 and HO_2 . These, in turn, are responsible for creating more NO_2 , which react with OH to generate HNO_3 . The latter is then transferred quickly through the water phase (high Henry coefficient) and dissociates to form detectable NO_3^- . In this context, OH reacts more readily with NO_2 , forming HNO_3 rather than with itself to create H_2O_2 .
- Between $R = 0.2$ – 0.6 , a reduction in the NO_3^- content is observed. The oxygen ratio decrease is responsible for a reduction in O , HO_2 , and O_3 species: all of which are precursors for converting NO to NO_2 . Because the reaction depletes 3 precursors at the same time, the production of HNO_3 drops quicker than that of HNO_2 .
- For $R = 0.6$ – 0.8 , the production of HNO_2 species declines because of the decrease of NO concentration, due to the depletion of O.
- Between $R = 0.8$ – 0.95 , NO_2^- is produced with competing mechanisms. In (Schmidt-Bleker et al., 2016), it was observed that the highest creation of N_2O was to be expected around 90 % nitrogen content, and is attributed to a rise in excited molecular nitrogen (N_2^*) species. This increase in N_2^* is responsible for the local maximum of O, and therefore as well for a local maximum of NO.
- At $R \leq 0.9$, the depletion of oxygen precursors is responsible for a reduction in the NO formation and thus both NO_2^- and NO_3^- formation.

Both Figs. 3 and 4 are in agreement that the optimal deactivation of *Pythium ultimum* occurs near $R = 0.95$. The plasma treatment over a duration of 30 min results in a SDB with a $\text{pH} > 4.5$; as explained earlier, although this might reduce somewhat the growth of pythium, it is not expected to be the main contributor. This finding also shows that the growth reduction of pythium is not dominantly attributed to the presence of NO_2^- , which has a toxic effect, since more NO_2^- is present at $R = 0.8$ than at $R = 0.95$. It can thus be presumed that the inactivation effect goes beyond the longer-lived species of NO_2^- and NO_3^- .

The antibacterial effectiveness of oxygen and nitrogen containing plasmas in many cases can be attributed to the generation of peroxynitrous acid (ONOOH) (Machala et al., 2012). Gaseous NO and NO_2 dissolve in water, contribute to the formation of NO_2^- and NO_3^- and acidic pH, and H_2O_2 and NO_2^- react in plasma treated water under acidic conditions to form intermediate ONOOH that eventually decays into $\cdot\text{OH}$ and $\cdot\text{NO}_2$ radicals responsible for the strong antibacterial effects. It is however typically produced at pH lower than 3 (Lukes et al., 2014), therefore an unlikely pathway at pH 4.5 in the SDB.

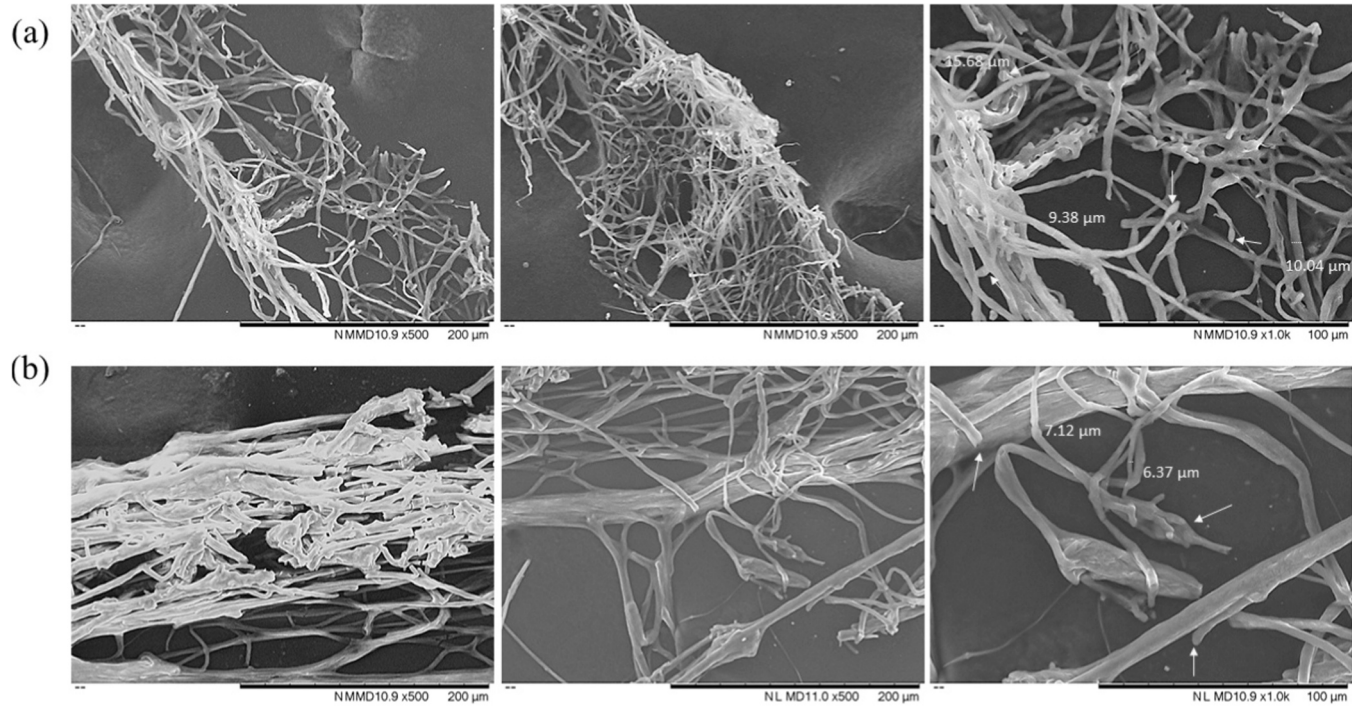


Fig. 5. Effects of plasma treatment on *P. ultimum* mycelia showing the impact on morphology, spores, and cell integrity for (a) untreated control sample, and (b) $R = 0.95$ (95 % N_2 , 5 % O_2) 30 min plasma treatment.

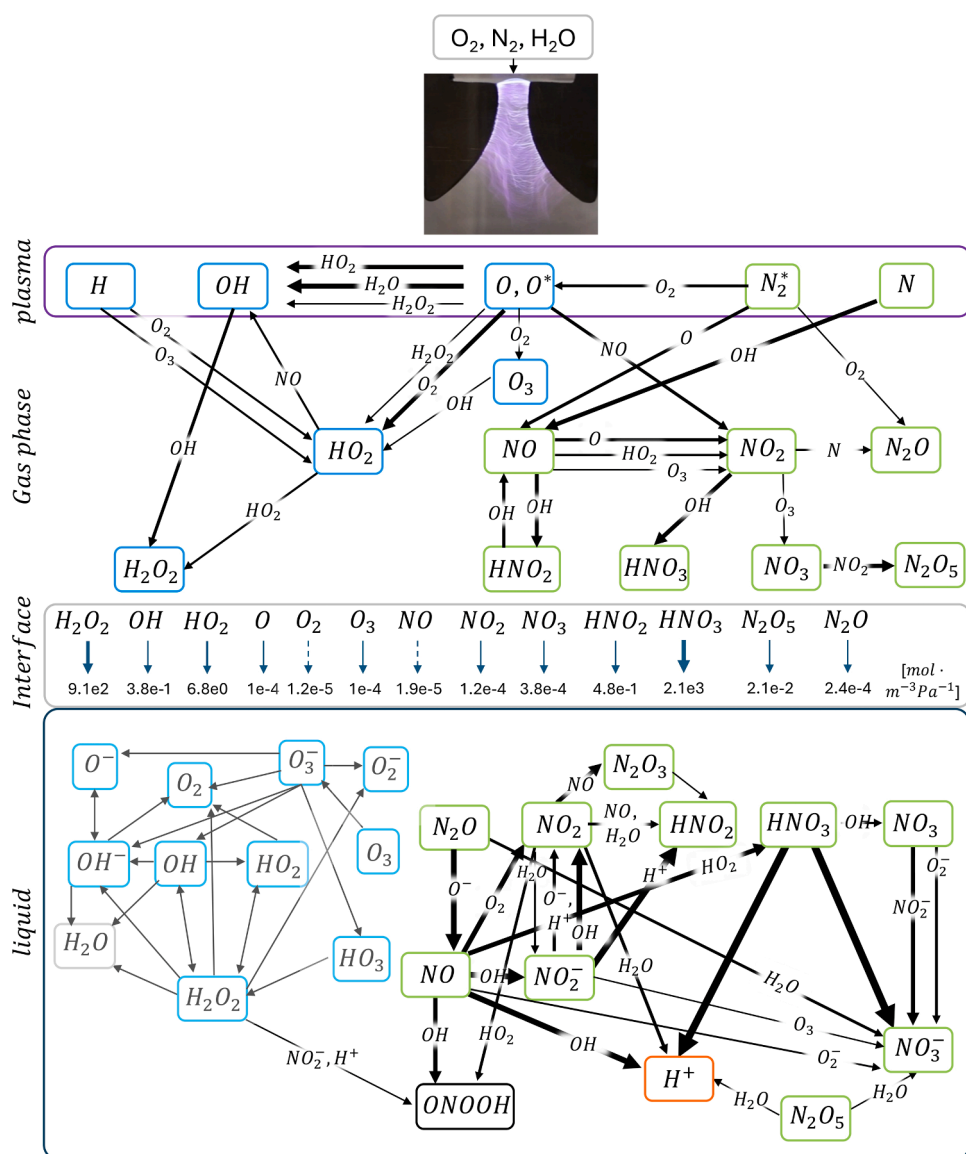


Fig. 6. Schematic diagram identifying important species generated as a result of predominant air plasma gaseous and aqueous reactions, including their transport from plasma to water driven by species-specific Henry's law solubility coefficients. The arrow thickness are indicators of orders of magnitude of the reaction rates. In the liquid phase, the details of arrow thickness related to reaction rates is kept for the nitrogen species and omitted for the OH derived species for simplification purposes.

In summary, plasma generates reactive species that attack the cell wall and cellular components (Sampaio et al., 2022). This causes severe damage that can lead to cell death. Oxidative stress caused by the plasma generated RONS can damage the cell membrane integrity, causing structural and functional damage to the cells and fungal hyphae (Wannicke and Brust, 2023). Disruption and morphological changes thus occur, in addition to the damage and leakage of the proteins and genetic components (DNA and RNA), leading to microbial inactivation. Although these arguments have been first proposed primarily in connection with bacteria, they have also been observed in the case of fungi (Niedzwiedz et al., 2019), as viewed earlier in the introduction, and are likely to extend to *Pythium ultimum*. Indeed, the SEM microscopic examinations confirms these mechanisms of cell wall erosion (etching), mass loss, hyphal breakage, all resulting in *Pythium ultimum* inactivation. Sporangium or oogonia containing structures terminating hyphae have been shown to be broken and significantly reduced after plasma treatment.

4. Conclusions

The research we report here constitutes an innovative laboratory-scale feasibility demonstration of cold plasma treatments using atmospheric pressure gliding arc discharges in $N_2 + O_2$ mixtures of differing ratios. Such treatments of *Pythium ultimum*-inoculated

water clearly demonstrate that the PTW, particularly that corresponding to $R = 0.95$, is capable of oomycete inactivation. Plasma treatment can thus contribute significantly to increase greenhouse agriculture production efficiency, where annual yield losses due to *Pythium* infections are substantial.

A second beneficial effect of cold atmospheric pressure plasmas applied to various R mixtures is that resulting RONS, by their very definition, include stable chemical compounds of atomic nitrogen, in other words fixated nitrogen, that can act as fertilizer for greenhouse-cultured crops. Thus, cold atmospheric pressure plasma technology is a potentially very powerful new tool for greenhouse culture in both respects, inactivation of pathogenic microorganisms, and fertilization. The advantages lie in the intrinsic sustainable properties of plasma technology, as it is based on locally available, abundant, and low-cost resources, namely air, electric power and water. Its carbon footprint and cost are mostly defined by the source of electricity and its infrastructure's impact. Plasma can potentially replace established conventional chemical insecticides and fertilizers, which are both expensive, and in cases of disruption of logistic chains, are of limited global accessibility, and are heavily polluting. In our work, we successfully showed that cold plasma treatment can be tuned to achieve the desired effect in *Pythium ultimum* inactivation for future use in greenhouse farming.

Our work's aim has been to identify optimal plasma chemical conditions to inactivate *P. ultimum* by adjusting the nitrogen to oxygen chemistry in a non-thermal gliding arc reactor. The reported data was acquired with tap water, inoculated distilled water or Sabouroud dextrose broth. Water conditions, based on pH, conductivity and dissolved solids, beyond these specific media may react differently. In addition, particulates in real greenhouse water may also act as radical scavengers. As ongoing work, matching plasma conditions with specific growth media for various crops is under research. Our investigation was limited to 3 selected plasma conditions for bio testing; further optimization, especially regarding process scaling is the subject of ongoing research. While in the present study, lettuce phytotoxicity of plasma treatment has not been studied, preliminary studies show great promise.

CRediT authorship contribution statement

Bédard Emilie: Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Côté Caroline:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Wertheimer Michael R.:** Writing – review & editing, Supervision, Conceptualization. **Tavares Jason Robert:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Larouche Élodie:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Data curation. **Abdelmalek Fady:** Methodology, Investigation. **Monette Julie:** Writing – review & editing, Validation, Methodology. **Généreux Mylène:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Watson Sean:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Reuter Stephan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Validation.

Author Contributions

SW performed plasma device design, plasma chemical characterisation and the pythium inactivation procedures. FA together with SW performed the hyphal mass measurements. FA performed the SEM measurements and analysis. First draft writing was done by SW and FA. Elisa Tests were conducted by EL. The paper strategy, writing and corrections were done by all authors.

Declaration of Competing Interest

J.M. works at GenV, a hydroponic greenhouse producer. The collaboration has no influence on the outcome of the results. The authors have no other conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.eti.2025.104222](https://doi.org/10.1016/j.eti.2025.104222).

Data Availability

Data will be made available on request.

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