



Deactivation of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* by novel cylindrical dielectric barrier discharge plasma: Maintaining biochemical composition in strawberry by *in-vitro* and *in-vivo*

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ABSTRACT

Strawberry fruits are slightly tender after harvesting, but they can normally fungal spoilage. The key strawberry fruits/plant fungus are *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*. There are many alternatives to conservative fungicides that can have a lower impact on humans and the environment, while maintaining the biochemical properties of strawberry fruits/plants. In this study, a multi-cylindrical dielectric barrier discharge plasma (MC-DBDP) system was employed to generate NO_x and O₃ gas, and plasma-activated water (PAW) by employing 100 % and 26.5 % duty cycle respectively. Study results showed that plasma generated reactive oxygen and nitrogen species (RONS) inactivate the *C. acutatum* at 2.87 and 3.57 respectively in 10 min, while *C. gloeosporioides* was found at ND. Two-way generated NO_x was used to inactivate the fungus, PAW-NO_x concentration was 250 μM and PAW-O₃ was 30 μM. *C. acutatum* decreased by 2.27 and 1.23 in PAW-NO_x and PAW-O₃, respectively, and *C. gloeosporioides* decreased by 1.23 and 1.42 in PAW-NO_x and PAW-O₃, respectively. Inactivation of the fungus was achieved by spraying and irrigating strawberry fruits and plants with PAW. PAW-NO_x not only controls the fungus but also improves biochemical composition of the other treatments (PAW-O₃) and control groups. At the same time, the necrotic effect induced by PAW-O₃ irrigation is greater when compared to control and PAW-NO_x.

1. Introduction

Anthraco-nose or fungal disease of strawberry (*Fragaria x ananas*) is caused by the genus *Colletotrichum*, specifically on strawberry leaves, fruits, and roots. Species such as *Colletotrichum acutatum*, *Colletotrichum fragariae* and *Colletotrichum gloeosporioides* mainly infect strawberries (Howard et al., 1992). *C. gloeosporioides* and *C. acutatum* are the most virulent fungal disease agents found on strawberries in Korea and European countries (Feliziani & Romanazzi, 2016; Nam et al., 2008, 2013). *Colletotrichum* infection affects various plants, for example, strawberries, olives, blueberries, citrus, etc. This *Colletotrichum* causes anthracnose diseases, causing leaf blight, crown root rot, and fruit rot (Azevedo-Nogueira et al., 2020). These *Colletotrichum* are capable of causing anthracnose disease in fruits, and have caused major losses in fruit storage in the recent years (Da Silva et al., 2020). The presence of

Colletotrichum in strawberry fruits after harvest is a major problem, and food shortages can be avoided by inactivating the fungus in these strawberry fruits after harvest. Fungal infections are a major problem for food safety by promoting mycotoxins, as they pose a significant threat to human and animal health. Such organisms include the following genera: *Colletotrichum*, *Fusarium*, *Penicillium*, *Rhizopus*, *Alternaria*, *Aspergillus*, *Botrytis*, and *Trichothecium*, these are largely contaminating post-harvested fruits and vegetables (Petriacq et al., 2018). Additionally, integrated management is required to control the disease in fruits or plants infected with *Colletotrichum* species *C. acutatum* and *C. gloeosporioides* in post-harvest conditions.

Various fungicides such as quinone external inhibitors (QoIs), dicarboximides (DCFs), methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs) are used to prevent *Colletotrichum* damage into fruits

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(Luo et al., 2022). While others synthetic fungicides are mancozeb, carbendazim, prochloraz and Tecto 60 (Chechi et al., 2019; Sengupta et al., 2020). Fungicides are not only effective in controlling the damage caused by *Colletotrichum* but are also toxic to human health and the surrounding environment (Singh et al., 2016), and sometimes fungicide-resistant varieties are produced (Tian et al., 2016). Continuous use of chemical fungicides causes resistance in plant phytopathogenic fungi, including *C. gloeosporioides* (Rathod, 2012); Therefore, the aim is to find new strategies to avoid fungicide resistance and to control phytopathogenic fungi (Salvatore & Ritenour, 2007). Non-chemical treatments for fruit and vegetable processing, such as nonthermal plasma, ozone and UV-C radiation, have shown promise in terms of safety. According to a Gumedde et al. (2018) study by ozone (gaseous form) and UV-C radiation play an important role in extending the shelf life and preserving the quality of strawberries. Ozone or UV is not good for long-term use indoors or outdoors, as it requires a lot of energy and can indirectly cause problems in the food or surrounding environment.

In general, a non-thermal plasma is a collection of electrons and ions produced in an electrically neutral, gas-phase medium. This plasma is used in biology and medicine to produce various types of RONS produced during electron-generated activity at temperatures of 0.8–3 eV and plasma densities of $3\text{--}5 \times 10^{12\text{--}16} \text{ cm}^{-3}$ (Choi et al., 2022). The use of non-thermal plasma has been shown to inactivate the bacteria (Borkar et al., 2023), viruses (Ashokkumar et al., 2023, 2024), cancer (Jaiswal et al., 2025), phytopathogenic fungi (Veerana et al., 2022), toxic by-products (Puligundla et al., 2020) and food sector (Pleslić & Katalenić, 2025). Various types of plasma devices such as Micro-DPD and Surface-DPD, Nanosecond Discharge Plasma and Jet Plasma are used to inactivate various types of pathogens (Ashokkumar et al., 2023, 2024; Choi et al., 2021). These plasmas have a number of beneficial properties, for example, they can be easily turned on and off, require little maintenance, and operate at ambient temperatures in dry conditions. Increase or decrease the RONS production due to the current and voltage modification.

After harvest, strawberries are contaminated with various pathogenic microorganisms, especially fungi. These fungi are inactivated by plasma gas exposure and plasma activated water (PAW). The plasma gas generated in the jet plasma inactivates 50 % of *Fusarium oxysporum* in paprika in the 90s. Furthermore, this inactivation depends on the power of plasma generation and treatment time, and they did not change the surface morphology, color, and firmness of paprika during plasma treatment (Go et al., 2019). Yang et al. (2023) reported DBD plasma activated water inactivates normal existing fungus and maintain the firmness, color quality, total soluble solids, malondialdehyde, vitamin C content and antioxidant activities of strawberry, this happens depending on PAW containing RONS (NO_2 , NO_3 , H_2O_2 , and O_2). Reactive species generated from plasma damage the cell wall and inner membrane of the fungus (Dasan et al., 2017; Jo et al., 2014). Some studies also suggest that plasma and UV radiation induce cross-linking with nucleic acids and oxidation of proteins and lipids (Dobrynin et al., 2009; Panngom et al., 2014).

Fungus inactivation in the field is important for ensuring crop health and food safety. Different kinds of synthetic or chemical fungicides are used to inactivate the fungus, and these fungicides indirectly promote the toxic byproducts to the food, environment, and humans. To avoid these types of problems we used PAW to inactivate the fungus as well as maintain food hygiene and reduce the environmental pollution. Several studies have shown that fungal infection negatively impacts plant growth and bio-chemical compositions. During the infection plant growth and bio-chemical compositions are reduced, some of the study indicated PAW enters into the leaves tissue through stomata and then inactivates the fungus (Zhang et al., 2014). If fungus infection is less, PAW induced growth of the plant (Puligundla et al., 2017), increases or maintains the biochemical and antioxidant properties. Some of the studies reported RONS highly inactive in the fungus cell rather than plant cell. Although both pathogens and plants have oxidative stress to

reduce, plasma-generated RONS species can increase the level of cellular RONS in plants, a phenomenon that limits antioxidant activity in microbial cells (Dobrynin et al., 2009; Gay-Mimbrera et al., 2016). In the present study, we inactivate *C. acutatum* and *C. gloeosporioides* using MC-DBDP generated gas and PAW (PAW- NO_x and PAW- O_3). After treatment [fungus inactivation in strawberry fruits and plants (leaves and root)], the fungus level and biochemical compositions were analyzed. Furthermore, to get a more comprehensive understanding of the nutrient dynamics influenced by plasma exposure, major nutrients including nitrate (NO_3^-), nitrite (NO_2^-), ammonium ion (NH_4^+) and phosphorus content were measured in strawberry plants/fruits for all categories. In addition, this study included a detailed analysis of RONS in both gas and liquid phases. This RONS assessment helped elucidate the underlying mechanisms involved in fungus inactivation. To sum up, this research provides an environmentally friendly and sustainable approach for effectively inactivating fungus in both strawberry plants and fruits while preserving their biochemical properties. The findings support the potential application of MC-DBDP as an alternative to conventional fungicides.

2. Materials and methods

2.1. Plasma source

In this study, two species of *Collectrodricum* sp (*C. acutatum* and *C. gloeosporioides*) were inactivated with NO_x and O_3 generated by multi-cylindrical dielectric barrier discharge plasma (MC-DBDP). The MC-DBDP consists of 4 pairs of brass electrodes, all electrodes covered with cylindrical quartz sleeves. All the electrodes are arranged in an alternating pattern, with each electrode connected to either a high-voltage or ground electrode. The electrode consists of 2 layers of cylindrical quartz sleeves; the outer quartz sleeve is 220 mm long and 30 mm in diameter and the middle quartz sleeve (inner) is 220 mm long and 6 mm in diameter. The distance between the high voltage and ground electrode is 2 mm, and between the two electrodes plasma was generated. The 1 LPM of ambient air was used to generate plasma from this device. This PAW was used to inactivate the fungus. Even though the reactor's configuration is the same, the dominant reactive species present will be different in duty cycle employed circuit in comparison to that without the duty cycle. In the duty cycle employed circuit, ozone is the major reactive species whereas the continuous circuit provides NO as the major species that is well described by the results of FTIR. PAW was created by introducing plasma gas NO_x (PAW- NO_x) and O_3 (PAW- O_3) separately into 1 L of tap water for 3 h. This PAW was used to inactivate the fungus and study the growth and development of plants in its presence.

In this study, we used a slightly modified MC-DBDP (Fig. S1 A) to inactivate the *Collectrodricum* Sp in strawberry fruits and plants (Fig. S2–S10). The duty cycle of the NO_x mode is 100 % and the O_3 mode is 26.5 % (on time: 90 ms, off time: 250 ms). This reactor was operated at a discharge voltage of 8.4 kV, a continuous resonant frequency of 21 kHz sinusoidal wave, and a power of 31.4 W to produce NO_x (Fig. S1 B). The O_3 mode is used to produce O_3 in the following conditions as follow, the discharge voltage of 8.63 kV, a discontinued resonant frequency of 20 kHz sinusoidal wave, and a power of 2.70 W (Fig. S1 C). Eq. (1) was used to obtain duty cycle and the electrical power (P) was measured according to the voltage versus current curve via Eq. (2) and (3) (Dahal et al., 2024a). Wherever T is the period of the voltage curve (Fig. S1 D).

$$\text{Dutyratio}(\%) = \frac{\text{on time}}{\text{on time} + \text{off time}} \times 100 \quad (1)$$

$$E = \int_0^T v(t) \times i(t) dt \text{ [J]} \quad (2)$$

$$P = \text{Duty ratio} \times \frac{1}{T} \int_0^T v(t) \times i(t) dt \quad [\text{W}] \quad (3)$$

where T is the period of voltage, and $v(t)$ and $i(t)$ are the recorded voltage and current signals, respectively.

2.2. Plasma voltage and current, OES and gas FTIR

The digital oscilloscope was used to analyze the current and high-voltage waveforms of MC-DBD plasma which were measured using high voltage (P6015A, Tektronix, Beaverton, OR, USA) and current probe (P6021, Tektronix, Beaverton, OR, USA). Optical emission spectroscopy (OES) was used to measure the relative intensities of reactive species in the gas phase generated by cylindrical DBDs. The spectrum is measured with the help of HR4000CG-UV-NIR spectrometer, Ocean Optics (USA, Orlando, Florida), and this spectrometer is united with an optical fiber. MC-DBD plasma generated gaseous RONS (mostly NO_x , NO_2 , N_2O , HNO_3 , and O_3) were examined by Fourier-transform infrared spectroscopy (FTIR) device (Matrix-MG5, Bruker, MA, USA), and the spectrum range was between 200 to 1100 nm.

2.3. Characterization of plasma-activated water

The pH, electrical conductivity, oxidation-reduction potential, total dissolved solids, salinity, and dissolved oxygen were measured with the help of Orion Versa Star Pro Benchtop pH meter (Thermo Scientific, Waltham, Massachusetts, United States). NO_2^- was measured in the PAW- NO_x , PAW- O_3 , and pesticides using Griess reagent assay (Merck). For the evaluation of NO_3^- in PAW- NO_x , PAW- O_3 , and pesticides (PC), a progressive method was used. To begin with, 5 mg of Zn powder was mixed in the sample and kept for 15 min at room temperature to reduce NO_3^- to NO_2^- . Griess reagent was used to measure the total NO_2^- present in the sample (Lamichhane et al., 2024). To get the exact amount of NO_3^- in the sample, the concentration of NO_2^- calculated before the reduction was subtracted from the total concentration of NO_2^- .

2.4. Spore inoculum preparation

Two endophytic fungi associated with strawberry anthracnose species of *C. gloeosporioides* and *C. acutatum* were grown in sabouraud dextrose (SD-MB-Cell) and plates incubated at 25 °C for 7 days. After 7 days 0.5 cm² of grown fungus was transferred to 50 mL aqueous sterile SD, then cultured under constant conditions for 7 days. Then, it was filtered with sterile gauze to obtain the spore stock suspension and then the broth solution was filtered in a two-layer filter cloth. The final concentration of the spore suspension was 1×10^5 spores/mL, determined by the hemocytometer chamber counting method. These two species was a gift from Professor Kyungsoo Park, Department of Electrical and Biological Physics, Kwangwoon University, Seoul 01897, Republic of Korea.

2.5. Inactivation of fungal spores by plasma gas and PAW

In this experiment, two types of plasma sources (NO_x exposure and O_3 exposure) were used to generate a broad spectrum of long-lived RONS, as shown in supplementary figure (Fig. S1). Two types of plasma device-generated long and short-live species inactivate *C. acutatum* and *C. gloeosporioides*. In the first treatment, the plasma gas was directly exposed to the fungal spores and the second plasma gas was injected into an aqueous solution. After that these solutions were used to inactivate the fungal spores (Fig. S1). In direct gas exposure tests, the experimental approach was used to direct gas (NO_x and O_3) exposure to fungal spores up to 2 h. The 10^4 spore solutions were deposited on the surface of the glass coverslips to assess the effectiveness of a gas-phase treatment. Plasma gas was exposed to the coverglass surface containing fungus spore at different timing intervals (0–2 h). After plasma gas

exposure added 1 mL of saline, mix well, and collect the spores and then the mycelium cells were counted (spread plate methods). The second is plasma-activated water (PAW), PAW is created by two types of exposure first NO_x exposure and second O_3 exposure. Two liters of distilled water (DW) were taken into the plastic tank and 1 LPM of ambient air was injected to generate NO_x and O_3 up to 3 h. The 10^4 spores were inoculated into 1 mL of different concentrations of NO_x and O_3 was added and then incubated for up to 2 days. After different timing intervals, we checked the fungus inactivation level using a spread plate mycelium colony counting. Plasma fungus decontamination efficacy was compared to negative control and positive control (2 % NaOCl).

2.6. Inoculation of the fungus into strawberry fruits and then inactivation by PAW

Strawberry fruits were cleaned with 0.1 % NaOCl for 2 min, followed by 2 min of sterile distilled water (DW). Excess water was removed by placing them inside a clean bench for 20 min. Then 50 μL (10^{-5} CFU/mL) of fungus was added, waited for 20 min for the fungus to adhere to the surface of the strawberry, and then inactivated using PAW. Fungus-infected strawberry was immersed into the 500 mL of beakers containing 100 mL of PAW (in treatment) or DW (in control) at different timing intervals 0, 30, 60 s, and 4 and 8 min. At the same time, we analyzed the fungal inactivation for up to 1 min using PAW spray. During the experiment, maintained positive control (fungus infection but without DW washing), positive control (DW washing), negative control (no fungus), and both treatment (PAW- NO_x and PAW- O_3). After treatment (5th days), 10 g of fungus muscles were removed and the mycelium colony was analyzed.

2.7. Inactivation of normal/artificially existing or infecting fungus in the strawberry fruits by plasma gas and different kinds of PAW and others

To remove the normal existing fungus in strawberry fruits by a plasma gas exposure (0, 4, 8, 16 and 32 min), PAW and other acidic acid water (immersed in to 1 min). Washing is essential, the washing may promote excessive moisture and this can encourage mold and spoilage of strawberries. To avoid this type of problem and increase the shelf-life time of the strawberry we tried PAW washing. After purchasing strawberries (*Fragaria × ananassa* Duch), they are immersed into 100 mL of various treatments conditions up to 1 min, the treatments being as follows: control without washing (CWW), distilled water (DW) washing, distilled water spraying (DWS), NO_x washing and NO_x spraying (NO_x -S), O_3 washing and O_3 spraying (O_3 -S), O_3 acid water (O_3 AW: 1 mM of HCl was added into 1 L of tap water and then 3 h Ozone was injected and pH is 3.1) washing and O_3 acid water spraying (O_3 AWS) was carried out. Immersed strawberry is kept for up to 1 min removed dry and maintained at room temperature for up to 4 days. The growth of the fungus on the strawberry was then measured visually (naked eye). At the same time, we analyzed the inactivation of fungi (50 μL at 10^{-5} CFU/mL) in strawberry wounds and without wounds by plasma gas (NO_x and O_3) exposure experiments at different time intervals (2, 4, 6, and 8 min). The distance between the plasma gas outlet and the strawberry is 1 cm.

2.8. Inactivation of artificially and normally existing fungus in strawberry plants

Initially, the strawberry plants were purchased from local market and plants were seeded into the field (Kwangwoon University, 20 Gwangun-ro, Nowon-gu, Seoul, South Korea) and grown for up to 30 days; thereafter, all plants were divided into three groups (2 treatment groups and one without treatment). Each group design is in 3×2 , which means two PAW treatments (PAW- NO_x and PAW- O_3 ; sprinkling) and without PAW treatment (tap water sprinkling). In detail, *C. acutatum* and *C. gloeosporioides* infected leaves were treated with PAW (PAW- NO_x and PAW- O_3) and tap water (control), by sprinkling. Infection was created

before treatment, the leaf surface (three sets of trifoliate leaves) was lightly removed to induce disease, and the fungus was inoculated onto the leaf surface at a concentration of $10 \mu\text{L}$ (10^{-5} CFU/mL/plant). After 7 days, the fungus will grow and enter the leaves. Seven days after the fungus infection started the inactivation of fungus using PAW (PAW- NO_x and PAW- O_3) and TW (for control) sprinkling, and for experiment control purposes PAW (PAW- NO_x and PAW- O_3) irrigation without fungus infection whether PAW irrigations enhanced the plant growth or not. PAW sprinkling and irrigation were attained every day at two times (each plant 100 mL), and after 30 days have analyzed the different kinds of physical-chemical parameters. Concentration of the NO_x in the PAW- NO_x and PAW- O_3 is $50 \mu\text{M}$. During fungus inactivation in the field, the temperature is $24 \pm 2^\circ\text{C}$, the humidity was $75 \pm 5\%$, and the light period was 14 ± 2 h light and 10 ± 2 h dark.

2.9. Biochemical compositions of strawberry leaves, root and fruits

2.9.1. Determination of electrolyte leakage (EL) and relative water content (RWC)

EL was measured by slightly modified Wu et al. (2019) methods. In brief, 50 mg of fresh strawberry leaves were cut into small pieces and immersed into 10 mL of DW and slightly shaken at 50 rpm for 24 h at 22°C room temperature. After 24 h shaking measured initial electrical conductance (EC1) and afterward, the samples were autoclaved at 120°C for 20 min, cooled down, and measured electrical conductance (EC2). EL was analyzed based on Eq. (4). The RWC have analyzed slightly modified methods based on Bolat et al. (2022) and Javed et al. (2024). In detail, RWC was analyzed fungus-infected strawberry leaves were treated at PAW and non-treated fresh leaves were used. The 0.5g of leaves (Fresh weight-FW) were immersed in 1.5 mL of distilled water at 22°C room temperature overnight in dark conditions, and excess water was removed using tissue paper and then measured turgid weight (leaves swollen or distended due to absorption of water). After turgid weight (tw) measurement strawberry leaves were oven-dried at 65°C for 48 h, and dry weight (DW) was measured. RWC was analyzed by Eq. (5).

$$\text{EL (\%)} = (\text{EC1})/(\text{EC2}) \times 100 \quad (4)$$

$$\text{RWC (\%)} = (\text{FW}-\text{DW})/(\text{tw}-\text{DW}) \times 100 \quad (5)$$

2.9.2. Proline content

A hundred mg of fresh strawberry leaves were grind with the help of liquid nitrogen and then 5 ml of 3 % sulphosalicylic acid was used to homogenize the leaves. Homogenous was centrifuged at 5000 rpm for 20 min at 4°C . A 100 μL of supernatant was added into a 100 μL glacial acetic acid and 100 μL of acid ninhydrin, and then the mixture was boiled at 100°C for 60min and suddenly cooled down at the ice. After cooling add 300 μL of toluene to the mixture and mix well and wait for 5 min. Free toluene (supernatant of the mixture) content was measured at 520 nm and L-proline was used as a standard Zhang et al. (2018).

2.9.3. Total phenolic content (TPC)

The TPC content in the fresh strawberry leaves was measured based on Swain and Hillis (1959) methods. Chopped 500 mg of fresh strawberry leaves grind in liquid nitrogen and an additional 1.25 mL of methanol and homogenate were centrifuged at 5000 rpm for 20 min at 4°C . TPC reaction mixture contains 0.8 mL of sodium carbonate (2 %), 1 mL of Folin-Ciocalteu reagent (10 %), 180 μL of distilled water, then added 20 μL of supernatant of the plant-extracted. The mixture was incubated at room temperature for 2 h. While the mixture was measured at 725 nm. TPC content of the leaves was expressed on mg per gram of FW, which is found based on gallic acid equivalent.

2.9.4. Chlorophyll content

Chlorophyll content in strawberry leaves was analyzed before and after PAW irrigation. A 0.5 g of fully expanded fresh leaves was taken and cleaned with sterile distilled water, and then 5 mL of 80 % acetone was added into a 50 mL conical tube and mixed well. The tube was covered with aluminum foil to avoid light and kept in a dark place at room temperature until fully transparent leaves were visible (up to 3 days). After incubation extracted liquid was filtered at $0.25 \mu\text{M}$ size. A 200 μL volume of filtrate liquid was used to measure chlorophyll *a*, *b* and total chlorophyll (*a+b*), this chlorophyll was measured at absorption wavelengths of 646 nm, and 663 nm respectively. The amount of chlorophyll was calculated based on the below equation of 6–8 (Lichtenthaler, 1987).

$$\text{Chlorophyll } a = (12.27 \times A_{663.2}) - (2.79 \times A_{646.8}) \quad (6)$$

$$\text{Chlorophyll } b = (21.50 \times A_{646.8}) - (5.10 \times A_{663.2}) \quad (7)$$

$$\text{Total Chlorophyll} = \text{chlorophyll } a + \text{chlorophyll } b \quad (8)$$

2.9.5. Total soluble proteins and carbohydrate

Strawberry leaves were cleaned with distilled water, 500 mg of leaves were frozen with liquid nitrogen, and then dissolved in 1 mL of PBS. The mixture was then centrifuged at 15000 rpm for 15 min. Protein was analyzed using the centrifuged supernatant and bovine serum albumin by DC protein assay (Bio-Rad, Hercules, CA, USA). Carbohydrates in strawberry leaves were assayed using anthrone, and glucose was used as a standard (Layne, 1975).

2.9.6. Nutrient content

Ammonium ion (NH_4^+), NO_2^- and NO_3^- and phosphorus were analyzed in strawberry root and fruits.

2.9.6.1. Ammonium ion (NH_4^+) content in strawberry root. The mixture of 10 mg dried root powder and 1 mL DW was shaken well and centrifuged at 12,000 g for 5 min. 0.1 mL of supernatant was allowed to mix with 0.4 mL of DW, 2.5 mL of phenol-sodium nitroprusside, and 2.5 mL of alkaline hypochlorite. Subsequently, the mixture was incubated for 30 min at room temperature and absorbance was measured at 635 nm using the plate reader. The incubated mixture results in a blue color. This process known as the Phenol-hypochlorite method is detailly explained in the previous study (Dahal et al., 2024b). The ammonium sulfate was used as the standard to form the calibration curve.

2.9.6.2. Evaluation of nitrite (NO_2^-) and nitrate (NO_3^-) in PAW, pesticides, strawberry root and fruit. To evaluate the NO_2^- content in the root of strawberry, 3 mL of hot (80°C) DW was added to 500 mg of grounded dried root and heated for 15 min. This solution was mixed with 2 mL DW and shaken followed by filtering it. The detailed information of this method is explained in Nerdy and Putra (2018). This filtrate was mixed with an equal volume of Griess reagent and incubated for 30 min at room temperature followed by the measurement of absorbance at 540 nm using a plate reader. The concentration of NO_2^- in fruit extract was also similar to the extract of the root of a strawberry plant (Bahadoran et al., 2016).

The concentration of NO_3^- was calculated using the salicylic acid method (Zhao & Wang, 2017). Approx 100 mg of strawberry root was frozen in 2 mL of liquid nitrogen. The resulting powder was mixed with 1 mL DW afterward. The mixture was then subjected to heating at 100°C for 20 min followed by centrifugation for 10 min. A 0.1 mL aliquot of the supernatant was mixed with 0.4 mL of salicylic-sulphuric acid (5 g salicylic acid in 100 mL of sulphuric acid) and incubated at room temperature for 20 min. Subsequently, 9.5 mL of 8 % sodium hydroxide was added to the mixture, allowing it to cool due to the heat generated by the reaction. The absorbance of the sample was measured at 410 nm using a

plate reader. In the case of fruit extract, NO_3^- was evaluated using the same method applied for measuring NO_3^- in PAW- NO_x , PAW- O_3 , and PC.

2.9.6.3. Phosphorus concentration in strawberry root and fruit. The concentration of phosphorus in the root of the strawberry plant was analyzed using the vanadomolybdo-phosphoric colorimetric method (Mukhtar, Manzoor, Gul, Zafar, & et al, 2020; Ryan & Estefan, 2001). To begin with, 100 mg of the dried root was grinded to powder. 5 mL mixture of concentrated nitric acid and perchloric acid in the ratio of 2:1 was mixed with grinded powder and then heated at a temperature of 200 °C in a hot magnetic stirrer (DLAB MS7-H550-S). The clear solution obtained after heating is diluted 4 times and filtered using Whatman filter paper No. 42. The filtrate was then subjected to vanadate molybdate reagent in equal volume and the exact concentration was calculated similarly to a previous study (Dahal et al., 2024a). Furthermore, in the case of fruit extract, equal volume of fruit extract was mixed with ammonium vanadate molybdate reagent, and the absorbance was measured at a wavelength of 410 nm using a plate reader (BioTek Synergy HT, Agilent Technologies) after 30 min of incubation at room temperature.

2.9.7. Statistical analysis

All tests were carried out using three independent sample replicates. The error bars represented the standard deviation from the mean values. Statistics were computed using Dunnett's/Tukey's various contrast analyses with two-way ANOVA via the PRISM software (version 8.0). The data were represented as follows: * indicated $p < 0.05$, ** indicated $p < 0.01$, and *** indicated $p < 0.001$.

3. Results

3.1. Plasma characterization

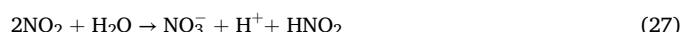
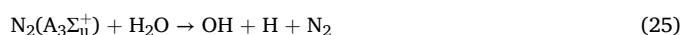
Both plasma modes (NO_x and O_3) discharged RONS were analyzed by OES techniques (Figs. S1 E, F). The N_2 SPS band emission is very dominant in NO_x mode and less in O_3 mode and also in both modes produced the first negative species. In this device, RONS production was increased by increasing the surface temperature of the quartz tube, with higher reactive species production attained at a higher temperature (450 K) and lower production in during reducing the temperature, and Ozone emission could be achieved by maintaining the quartz surface at a lower temperature. Ozone and NO cannot coexist at high temperatures because they require different temperatures to form (Kim et al., 2023). Gas FTIR in NO_x mode can detect various chemical species such as NO, NO_2 , and N_2O through the following reactions (Equations (9)–(14)) (Fig. S1 G), and HNO_3 , N_2O , O_3 , and NO_2 formed in O_3 mode can be detected through the following reactions (Equation (15)–(24)) (Fig. S1 H).



3.2. Physicochemical characteristics of PAW

Plasma gas (2 h) was injected into the water to form different kinds of RONS during the bubbling system, due to this RONS physicochemical conditions are changed particularly, pH, ORP, conductivity, salinity, TDS, and DO in tap water (TW) depending on NO_x and O_3 mode (Fig. 1A–L). We mainly inspected the amounts of reactive nitrogen species ($\text{NO}_2^-/\text{NO}_3^-$) in the TW, this reactive species produced around 250 μM in NO_x mode and 30 μM in O_3 mode. Plasma mode obtained NO_x concentration was diluted at different concentrations and checked the physicochemical and fungus inactivation. The pH of the distilled water is 6.67 and reduced the pH from 6.67 to 3.90 ± 0.05 in NO_x mode (Figs. 1 A) and 6.67 to 3.77 ± 0.03 in O_3 mode (Fig. 1 G) and, the pH concentrations reduced when increasing the NO_x concentration. ORP in the NO_x mode from 254.44 to 443.73 ± 1.63 mV (Fig. 1 B) and O_3 mode 248.70 to 372 ± 0.92 mV (Fig. 1 H). Conductivity in the NO_x mode from 31.37 to 683.70 $\mu\text{S}/\text{cm}$ (Fig. 1 C) and O_3 mode 20.55 to 331.45 ± 4.56 $\mu\text{S}/\text{cm}$ (Fig. 1 I), while TDS concentrations in the NO_x mode 31.92 to 249.38 ± 8.69 ppm (Fig. 1 E) and O_3 mode 9.71 to 120.5 ± 1.27 ppm (Fig. 1 J). Salinity in the NO_x mode from 0.038 to 0.31 psu (Fig. 1 D) and O_3 mode 0.016 to 0.025 ± 0.001 psu (Fig. 1 K) and also another parameter like DO in the NO_x mode from 1.01 to 1.79 mg/L (Fig. 1 F) and O_3 mode 1.90–2.19 mg/L (Fig. 1 L).

Here, the duty ratio plays an important role in plasma device (turns on and off the plasma discharge) ensuring a sustainable generation of ozone species (Fig. S1 D) (Dhakal et al., 2024). RONS production in gas space (Eqs. (9)–(24)) and RONS production in aquatic space (Eq. (25)–(32)) are given below. In agriculture the inactivation of fungi in the strawberry fields, different kinds of pesticides and pesticides induced toxic byproducts in strawberry plants, fruits and, also water and soil pollution. Plasma gas is injected into the water and produces a PAW; this PAW contains different kinds of RONS. The plasma generated PAW reduce the toxic effect at the same time reduce the fungus. Production of RONS in the water may be following these reactions, the reactions are followed. Excited nitrogen molecules in the metastable state $\text{N}_2(\text{A}_3\Sigma_u^+)$ are formed when a nitrogen molecule collides with an electron as $\text{N}_2 + e^- \rightarrow \text{N}_2(\text{A}_3\Sigma_u^+) + e^-$. This nitrogen interacts with water, which in turn releases these water molecules at $\alpha_{\text{HO}} = 5 \times 10^{-14}$ cm^3/s to form N_2 and a hydrogen atom H (Herron, 1999), (Equation (25)). The water contains OH elimination is shown in Eq. (26), and the rate coefficient of $\alpha_{\text{H}_2\text{O}_2} = 6.83 \times 10^{-31}(\text{Tr}/T)^{0.8}$ $\text{cm}^6/\text{mol}^2/\text{s} = 1.78 \times 10^{-11}$ $\text{cm}^3/\text{mol s}$ in the air at $T = 300$ K (Forster et al., 1995), and the M is the neutral particles. Nitrous acid (HNO_2) may dissolve into water to form H and NO_2^- (Eq. (27) and (28)). The O_3 reacts with water containing OH and produces HO_2 (Eq. (29)) with a rate coefficient is $\alpha_{\text{OH}} = 7.3 \times 10^{-14}$ cm^3/s (DeMore et al., 1997). Ozone may also eliminate nitrogen monoxide (NO) into NO_2 (Eq. (30)) with a rate coefficient of $\alpha_{\text{NO}} = 1.8 \times 10^{-14}$ cm^3/s (Atkinson et al., 2004). While also producing various NO and HNO_3 in Eq. (31) and (32).



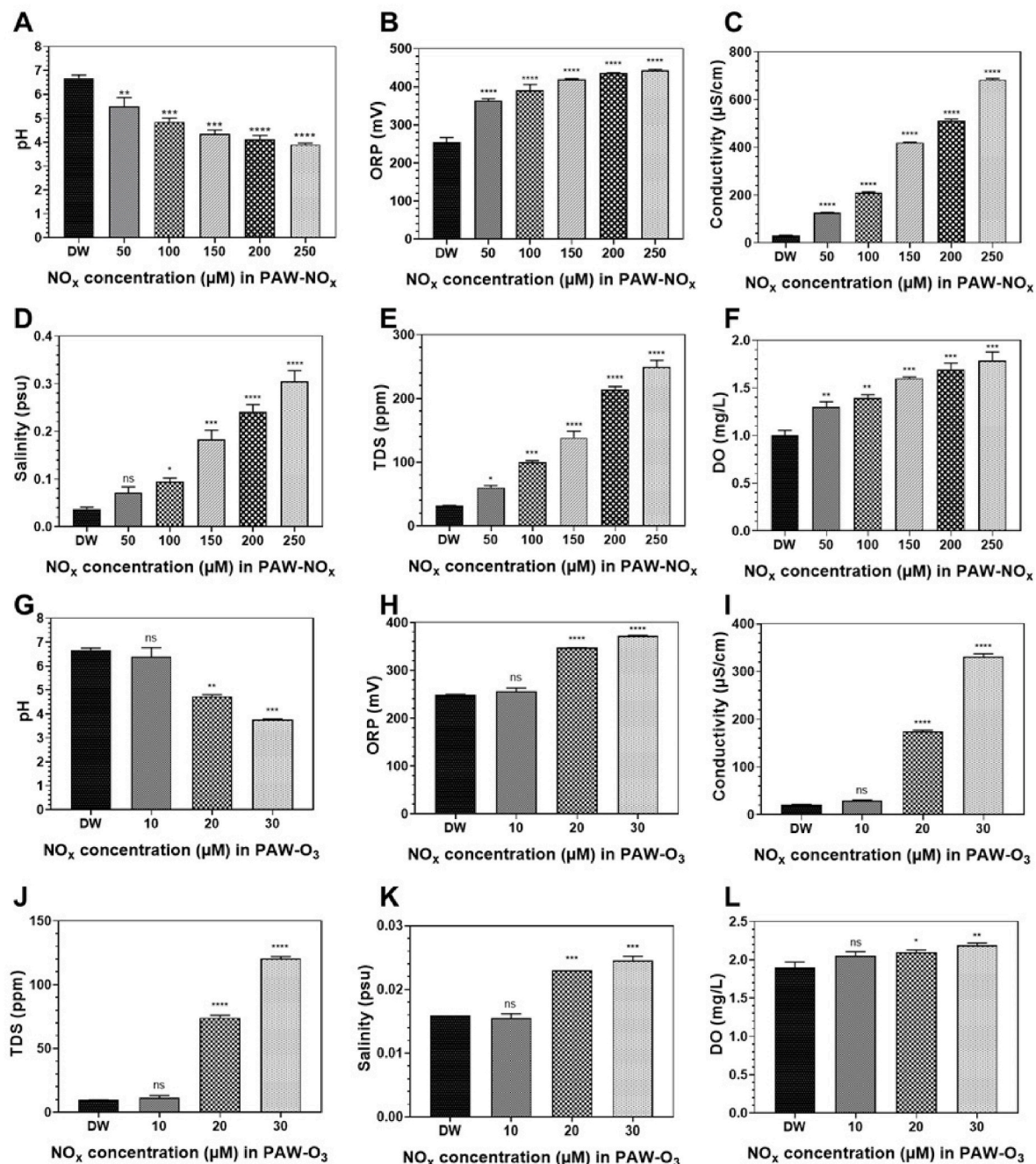
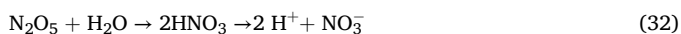
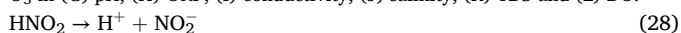


Fig. 1. Physicochemical characteristics of PAW: NO_x gas injected PAW-NO_x in (A) pH, (B) ORP, (C) conductivity, (D) salinity, (E) TDS, (F) DO; O₃ gas injected PAW-O₃ in (G) pH, (H) ORP, (I) conductivity, (J) salinity, (K) TDS and (L) DO.



3.3. Inactivation of *Colletotrichum* sp using plasma gas and PAW

Plasma gas exposure inactivated the fungus spore in 120 min (Figs. S2 A-C). NO_x gas exposure reduced the original *C.acutatum* log order reductions from 4.27 ± 0.16 to 1.4 ± 0.06 CFU/mL in 10 min,

after which there was no fungal growth. While *C. gloeosporioides* was reduced by NO_x gas from 3.49 ± 0.38 to 1.2 ± 0.14 CFU/mL in 5 min, there was no fungal growth. O₃ gas exposure reduced *C. acutatum* from 4.27 ± 0.16 to 0.7 ± 0.13 CFU/mL in 10 min (Fig. S2 A), and *C. gloeosporioides* from 3.49 ± 0.38 to 0.70 ± 0.03 CFU/mL in 5 min, with no fungal growth thereafter in 5 min (Figs. S2 B, C). Two species of fungi (*C.acutatum* and *C. gloeosporioides*) were investigated in different concentrations of PAW-NO_x generated by NO_x mode for 48 h (2 days) (Fig. 2A and B). *C. acutatum* was reduced by different concentrations of NO_x (50–250 μM), the fungal reduction was 0.81 ± 0.11 , 1.23 ± 0.08 , 1.72 ± 0.12 , 2.27 ± 0.17 and 2.75 ± 0.19 log reduction, achieved by control, 50, 100, 150, 250 μM of NO_x and 2 % of NaOCl, respectively (Fig. 2A). A concentration of 153.5 μM NO_x can inhibit *C. acutatum* by 50 % (Fig. 2E). The reduction of *C. gloeosporioides* was 0.57 ± 0.0 , 1.03

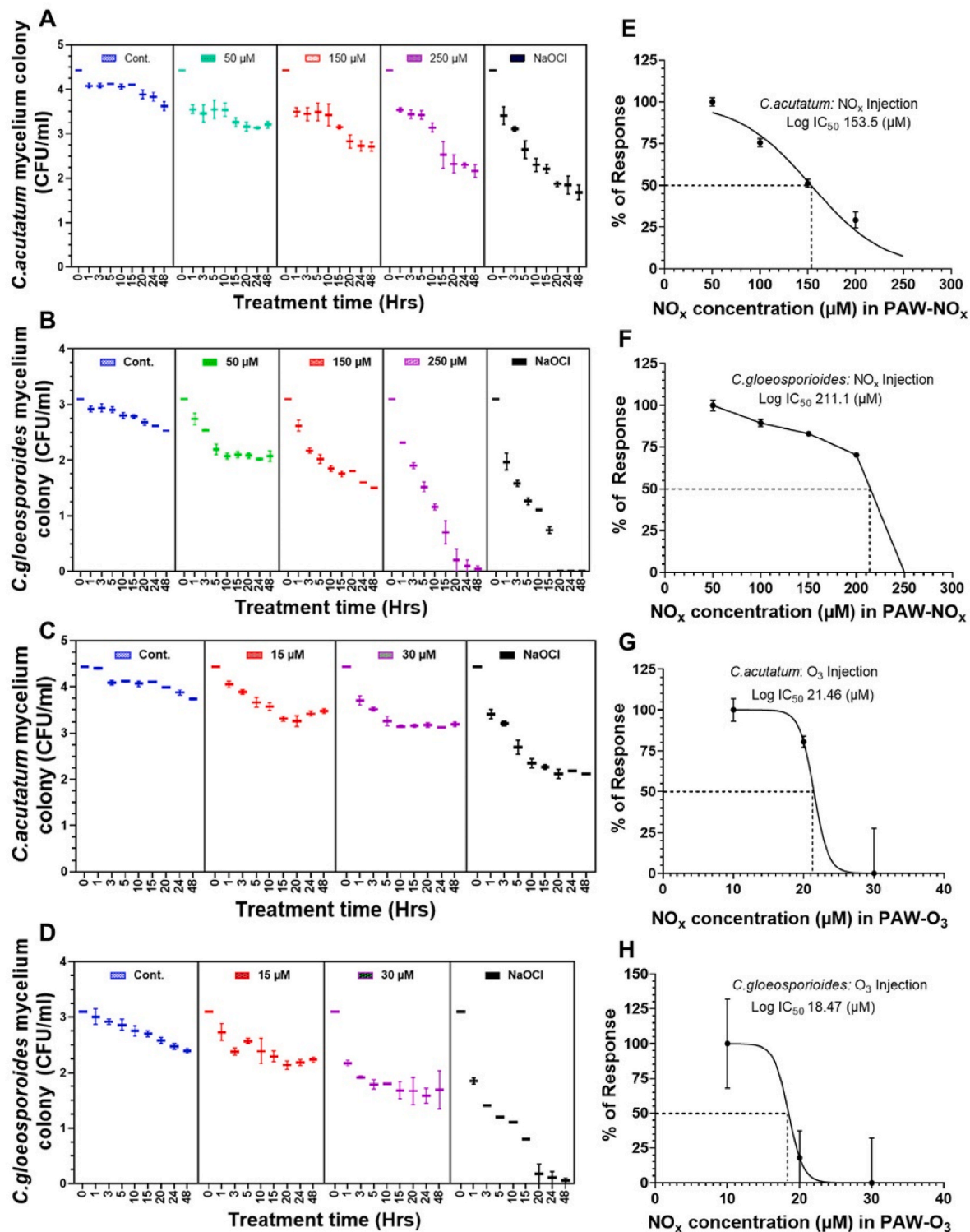


Fig. 2. Colletotrichum mycelium spore was inactivated at different time intervals with different NO_x concentrations generated by two different plasma devices and obtained the IC₅₀. Inactivation of mycelium at different NO_x concentrations obtained from PAW-NO_x; (A) *C. acutatum*, (B) *C. gloeosporioides* and PAW-O₃ by (C) *C. acutatum*, (D) *C. gloeosporioides*. 50 % inhibitory concentration (IC₅₀) of derivative with different concentration of NO_x: PAW-NO_x in (E) *C. acutatum*, (F) *C. gloeosporioides* and PAW-O₃ by (G) *C. acutatum* and (H) *C. gloeosporioides*.

± 0.1 , 1.60 ± 0.0 , 3.05 ± 0.06 and not detectable (ND), respectively, in the control, 50, 100, 150, 250 μM NO_x and 2 % NaOCl (Fig. 2B). The concentration of 211.1 μM NO_x achieves 99.9 % inactivation of *C. acutatum* in 48 h, 250 μM NO_x achieves 99.99 % inactivation of *C. gloeosporioides* in

24 h, and NaOCl in 20 h. Inactivation was induced by the O₃ mode generated NO_x (15–30 μM), the PAW-O₃ inactivation of *C. acutatum* and *C. gloeosporioides* induced by different timing intervals. *C. acutatum* inactivation was 0.69 ± 0.01 , 0.96 ± 0.06 , 1.23 ± 0.06 , and 2.31 ± 0.0 log order reduction in 48 h like control, 15, 30 μM of NO_x, and NaOCl, respectively (Fig. 2C) and the IC₅₀ was 21.46 μM (Fig. 2G). *C.*

gloeosporioides attain 0.71 ± 0.04 , 0.87 ± 0.06 , 1.42 ± 0.39 , and 3.05 ± 0.06 log order reduction are control, 15, 30 μM NO_x and NaOCl respectively (Fig. 2D) and IC_{50} is 18.47 μM (Fig. 2H), while in NaOCl attain 99.99 % inactivation in 24 h. PAW- O_3 enhanced the fungus efficacy rate up to 15 h and then not reduced.

Fig. S10 shows the concentration of nitrate (NO_3^-) and nitrite (NO_2^-) in PAW- NO_x and PAW- O_3 over a 60-min interval. The concentrations of NO_2^- and NO_3^- in PAW- NO_x were 49.02 and 103.39 ppm, respectively, and during PAW- O_3 treatment, they were 0.56 and 2.36 ppm, respectively. Equations (5)–(28) show the formation of these NO_x species. Although the short-lived species cannot be directly detected in the liquid, their production is confirmed by the accumulated concentration of NO_3^- and NO_2^- . Patel et al. (2024) reported that the amount of PAW- NO_x generated by cylinder DBD plasma remained stable for up to 20 days.

3.4. Inactivation of normal existing/artificially existing or infecting fungus in strawberry by plasma gas and PAW, and biochemical compositions

Plasma gas was exposed to containers containing strawberries for up to 32 min at different time intervals (0, 4, 8, 16 and 32 min) (Figs. S3 A, B). After that, the fungus growth was analyzed, up to 8 min fungus was slightly grown and then the fungus was not grown for 16 and 32 min (Figs. S3 A). The plasma significantly inhibited the fungus compared to the control. O_3 exposure produced a necrotic effect (black color) on the fungus in 16 and 32 min (supplementary file) (Figs. S3 B). On the fourth day, we analyzed the various biochemical compositions of strawberries. That is, the following biochemical compositions were observed in the NO_x and O_3 treatments for 16 min of treatment, respectively: proline 16.19 ± 2.42 – 15.65 ± 0.77 $\mu\text{M/g}$ FW, TPC 5.44 ± 0.36 – 5.07 ± 0.05 mg GAE/g FW, protein 1.19 ± 0.01 – 0.86 ± 0.09 mg/g FW, carbohydrate 2.57 ± 0.03 – 2.05 ± 0.05 mg/g FW, nitrate 135.78 ± 1.80 – 115.22 ± 5.49 mg/g FW, nitrite 2.92 ± 0.06 – 1.80 ± 0.01 mg/g FW and phosphorus 75.96 ± 0.68 – 52.13 ± 3.37 mg/g FW (Fig. 3A–G). The biochemical compounds in the control group are lower. The biochemical compounds in the treatment increase as the treatment time increases, and after 16 min the biochemical levels decrease. Supplementary Fig. 3 shown inactivation of normal existing fungus in strawberry, and artificially infected (Fig. S4) *C. acutatum* and *C. gloeosporioides* by NO_x and O_3 gas exposure (with or without wound). Both of them significantly control the fungus compared with control, but NO_x gas maintains the strawberry fruits freshness (less dark color and tissue damage). O_3 plasma gas exposure inhibits the fungus's growth in 2 min, but in NO_x , it takes 4 min. While the inner view (inside of the strawberry) showed a higher black color mold grown without treatment and less mold grown in the treatment group, between the group's NO_x treatment had a less black color (supplementary file) (Fig. S5). In the open field or Indore conditions little bit difficult conjugated, because need higher power and also produced environmental problem when using O_3 . Avoid this type of issues PAW are better. Different kind of PAW and other treatments control the fungus. The NO_x and O_3 -AWS are significantly inhibiting the growth of the fungus on strawberry compared with other treatment and control (Fig. S6 and 7).

Strawberry fruit's proline, TPC, protein, carbohydrate, nitrate, nitrite and phosphorus activity were determined without (control) and with PAW treatment (Fig. 3H–N). Proline level is 6.06 ± 1.40 , 12.12 ± 0.76 , 8.26 ± 1.40 and 9.14 ± 0.13 $\mu\text{M/g}$ FW in control, PAW- NO_x , PAW- O_3 , and PC, respectively (Fig. 3H). As well as TPC concentration is 4.22 ± 0.10 , 5.62 ± 0.16 , 4.27 ± 0.16 and 4.63 ± 0.36 in control, PAW- NO_x , PAW- O_3 , and PC, respectively (Fig. 3I). The protein concentration of strawberries has a 0.49 ± 0.01 , 1.11 ± 0.07 , 0.77 ± 0.01 , and 0.97 ± 0.01 mg/kg FW in the following treatments by control, PAW- NO_x , PAW- O_3 , and PC, respectively (Fig. 3J). While, the carbohydrate concentration is 1.09 ± 0.03 , 1.53 ± 0.06 , 1.15 ± 0.05 , and 0.99 ± 0.02 mg/kg FW in control, PAW- NO_x , PAW- O_3 , and PC, respectively (Fig. 3K). The

concentration of NO_3^- categories is illustrated in Fig. 3L, in control of the NO_3^- level in 86.26 ± 1.80 , 114.53 ± 6.27 , and 88.56 ± 5.13 , 70.73 ± 2.97 mg/kg of FW under the conditions of control and treatment of PAW- NO_x , PAW- O_3 , and PC respectively. Likewise, Fig. 3M shows the amount of NO_2^- present in the strawberry with the highest value of 2.32 ± 0.02 mg/kg FW in PAW- NO_x followed by 1.17 ± 0.03 , 1.28 ± 0.0 , and 1.07 ± 0.01 mg/kg FW in control, PAW- O_3 , and PC respectively. The presence of various RNS in PAW- NO_x and PAW- O_3 is responsible for the increment of NO_2^- and NO_3^- in strawberry fruits. However, PAW- O_3 has less RNS in comparison to PAW- NO_x due to which the increment in the value of NO_2^- and NO_3^- in PAW- O_3 is significantly low. Additionally, Fig. 3N shows that phosphorus concentration was higher (54.13 ± 1.44) in PAW- NO_x and slightly lower at 46.88 ± 0.19 by PAW- O_3 compared to PC (38.22 ± 1.73) and 44.13 ± 0.87 mg/kg FW without treatment. This may be due to the presence of RNS in PAW which is absorbed by fruits during the treatment process. These results suggest that PAW is suitable to maintain the nutrient content in fruits in comparison to PC.

3.5. Fungus inactivation in strawberry fruits by PAW and biochemical compositions

The post-harvest handling of strawberries is presented in Fig. 4. We used insecticides to control the fungal disease that develops on strawberries after harvest. Research is needed on the health risks of using this, as well as non-chemical post-harvest treatments. The non-thermal plasma produced gas or PAW, effectively inhibits the fungus propagation and maintains freshness of the strawberries. Inactivation of intently infected fungus by PAW- NO_x and PAW- O_3 , Fig. 4A indicated fungus inactivation based on the treatment time and conditions. After treatment growth of the mycelium was reduced with PAW- NO_x treatment compare to other treatment and control. A 1 min of disinfection of *C. gloeosporioides* with PAW- NO_x , the *C. gloeosporioides* mycelium was reduced from 5.12 ± 0.006 to 3.71 ± 0.08 (Fig. 4B) and the *C. acutatum* mycelium from 5.12 ± 0.017 to 3.95 ± 0.029 (Fig. 4C), and the loss of activity was less in the other treatments and the control. Fungus was effectively controlling the fungus at the same time up to 1 min control fungus and maintain the freshness of the strawberry, there after 1 min the cell are decay and released juicy. To avoid releasing the juicy from the strawberry, we used PAW spray to avoid juicy come out at the same time control the fungus (Fig. 4D). PAW spray also controls the fungus but compared to PAW washing less effective. The 1 min PAW washing reduced the fungus in the strawberry based upon the time and treatment conditions.

3.6. In-vivo inactivation of fungus in the strawberry leaves and biochemical compositions

After fungus inactivation in the strawberry by plasma gas and PAW, we analyzed fungus inhabitation in strawberry plants in the open field. During that time, we analyzed the following parameters different biochemical compositions in plant leaves (Fig. 5) and roots (Figs. 6 and 7). Chlorophyll content in strawberry leaves was directly related to photosynthesis, which was measured on day 30th after fungal infection/inactivation. Compared to the fungal infection in the control, chlorophyll a, b, and total content were found to decrease compared with PAW- O_3 and PAW- NO_x sprays (Table. S1 A). Furthermore, chlorophyll content in PAW- O_3 irrigation without fungal infection was found to be decreased compared to control and PAW- NO_x irrigation. PAW- O_3 irrigation results in decreased chlorophyll content due to necrotic effect when compared to all other treatments and control groups (Fig. S8). At the same time, we measured leaf length width, and root length after the with and without PAW treatment. PAW- NO_x treatment increases the leaf length (4.6–5.5 cm) width (4.9–5.4 cm), and root length compares to PAW- O_3 and control (Table. S1 B, C). In PAW- O_3 irrigation slightly reduced the leaf length width and root length compared to control and significantly less in PAW- NO_x . During the infection strawberry root was infected crown

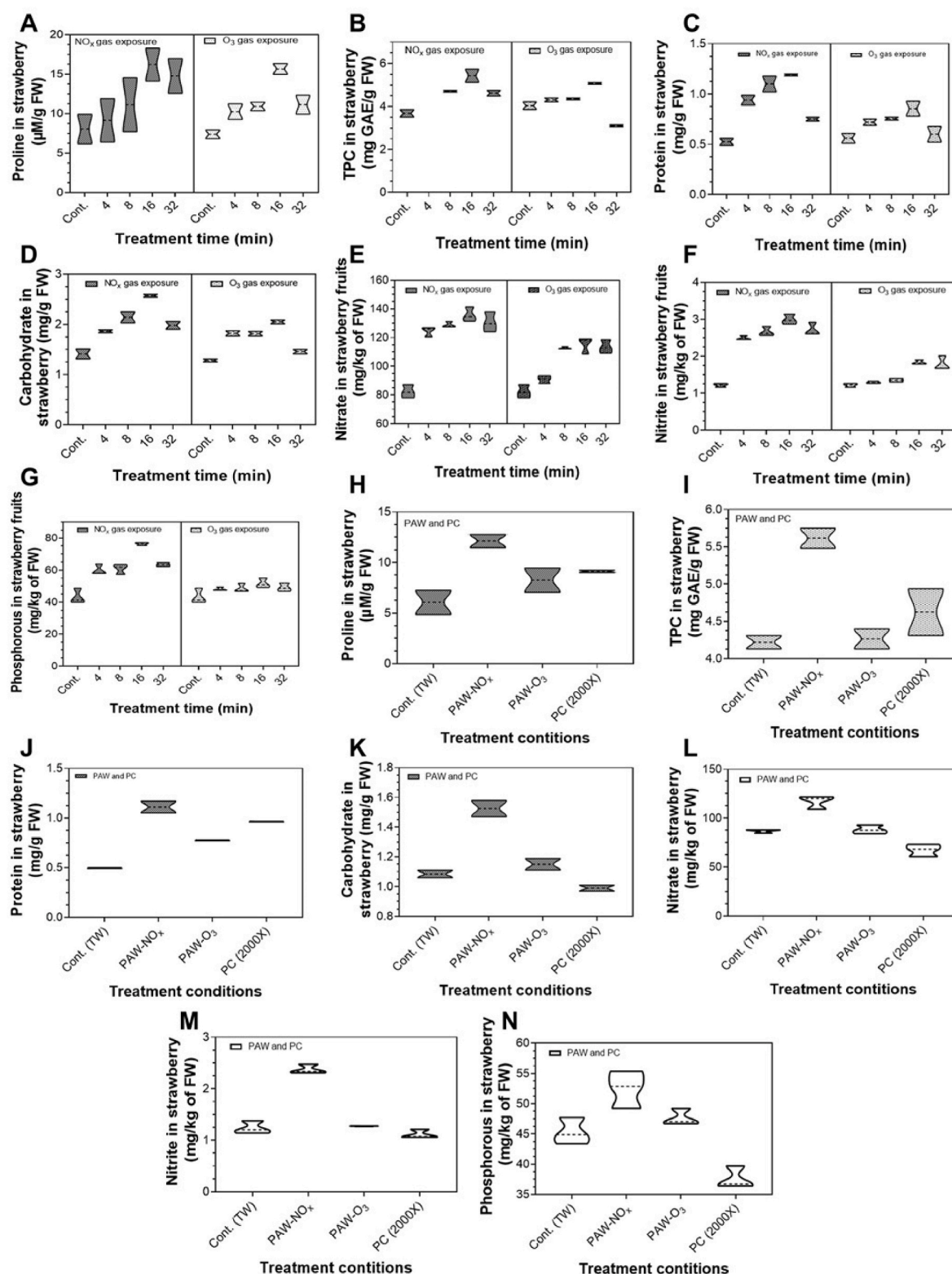


Fig. 3. Plasma gas exposure (NO_x and O_3) was used to inactivate normal existing fungus in strawberries fruits and improve or maintain biochemical composition compared to the control: biochemical composition as (A) proline, (B) TPC (C) protein, (D) carbohydrate, (E) nitrate, (F) nitrite and (G) phosphorous. PAW and pesticide (PC) control normally existing fungus in strawberries and maintain biochemical compounds resembling (H) proline, (I) TPC, (J) protein, (K) carbohydrate, (L) nitrate, (M) nitrite, and (N) phosphorous.

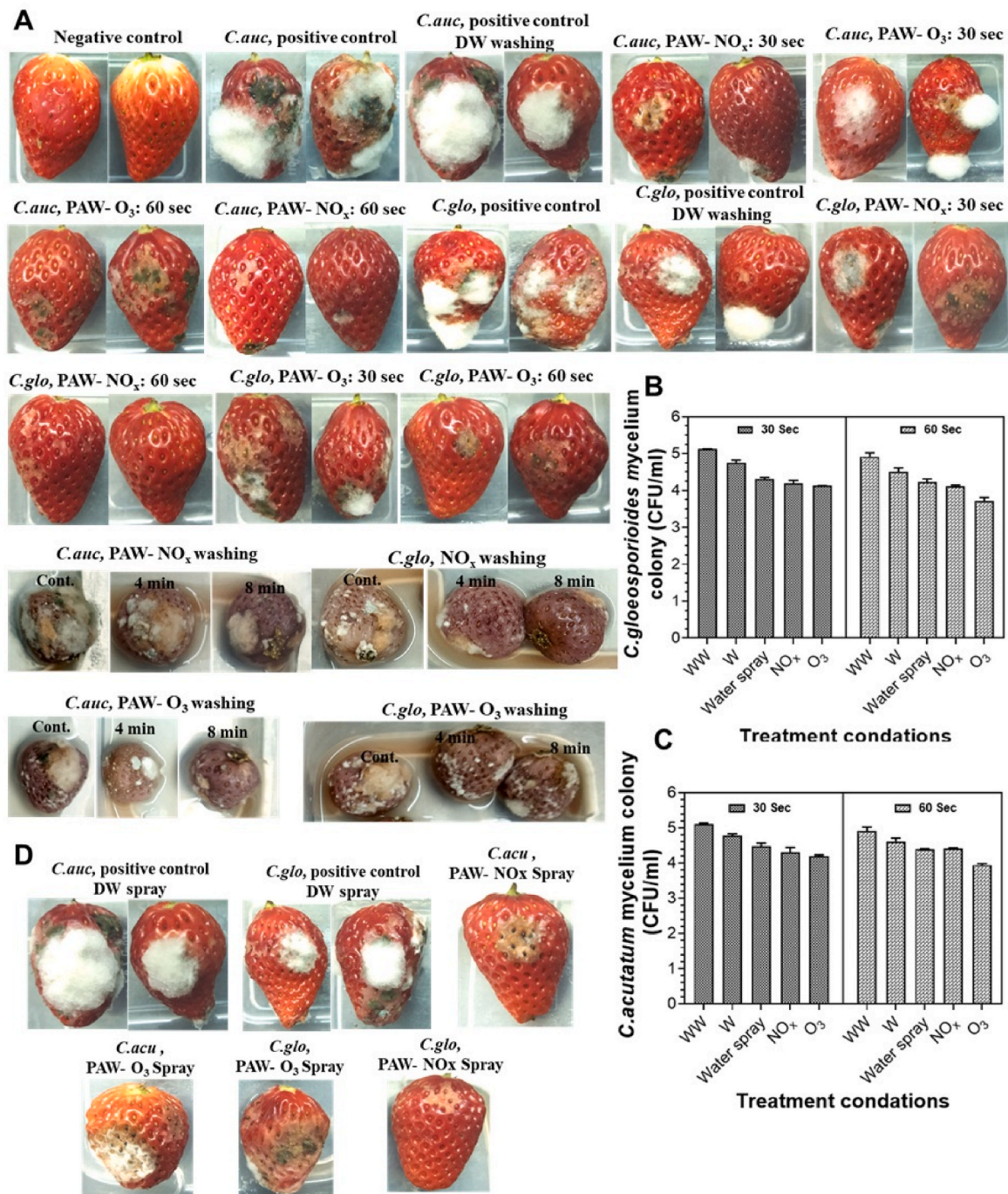


Fig. 4. Inactivation of *Colletotrichum* species on strawberries: (A) different fungus inactivation by washing and treatments with different PAW on strawberries, and mycelium colony (B) *C. gloeosporioides*, (C) *C. acutatum*, and (D) fungus inactivated by PAW spraying.

rot disease, after applying the PAW sprinkling and irrigation reduce the crown rot compare to control, between the treatment significantly reduce the crown rot disease in PAW-NO_x treatment (Fig. S9).

Biochemical parameter is depended on fungus infection and treatment conditions, the EC percentage are 20 ± 0.01 – 21.55 ± 0.44 %, 21.76 ± 0.55 – 22.35 ± 0.24 % and 22.59 ± 0.20 – 26.73 ± 0.13 % in PAW-NO_x, PAW-O₃ and control (TW) respectively (Fig. 5A–G). During the fungus infection, plants lass their cellular function, and increased ions escape. Simultaneously RWC are involved after the EC activation in after the fungus infection, the RWC values were 79.59 ± 3.90 – 80.75 ± 1.65 % in PAW-NO_x, 72.86 ± 2.01 – 76.97 ± 0.09 % in PAW-O₃, control 72 ± 1.24 – 73.78 ± 1.81 % (Fig. 5B–H). Amino acids, particularly

proline, reduce stress during fungus infection by enhancing the carbon and nitrogen source of the plant. Proline content in the plants is from 10.11 ± 0.25 – 17.16 ± 0.73 , 9.26 ± 0.48 – 11.68 ± 2.31 , and 2.16 ± 0.43 – 3.05 ± 0.97 $\mu\text{M/g}$ FW in PAW-NO_x, PAW-O₃, and control respectively (Fig. 5C–I). TPC content is from 10.20 ± 0.03 – 11.86 ± 0.55 , 8.63 ± 0.32 – 9.95 ± 0.13 , and 7.25 ± 0.07 – 8.24 ± 0.77 mg GAE/g FW in PAW-NO_x, PAW-O₃, and control respectively (Fig. 5D–J). Significantly maintains proline content in the PAW-NO_x compare to other treatment and control. The protein content is from 3.70 ± 0.47 – 5.05 ± 0.19 , 3.57 ± 0.09 – 4.38 ± 0.21 , and 2.81 ± 0.01 – 4.30 ± 0.06 mg/g FW in PAW-NO_x, PAW-O₃, and control respectively (Fig. 5E–K). PAW-NO_x irrigation contains a higher amount of protein than all other treatments. At the

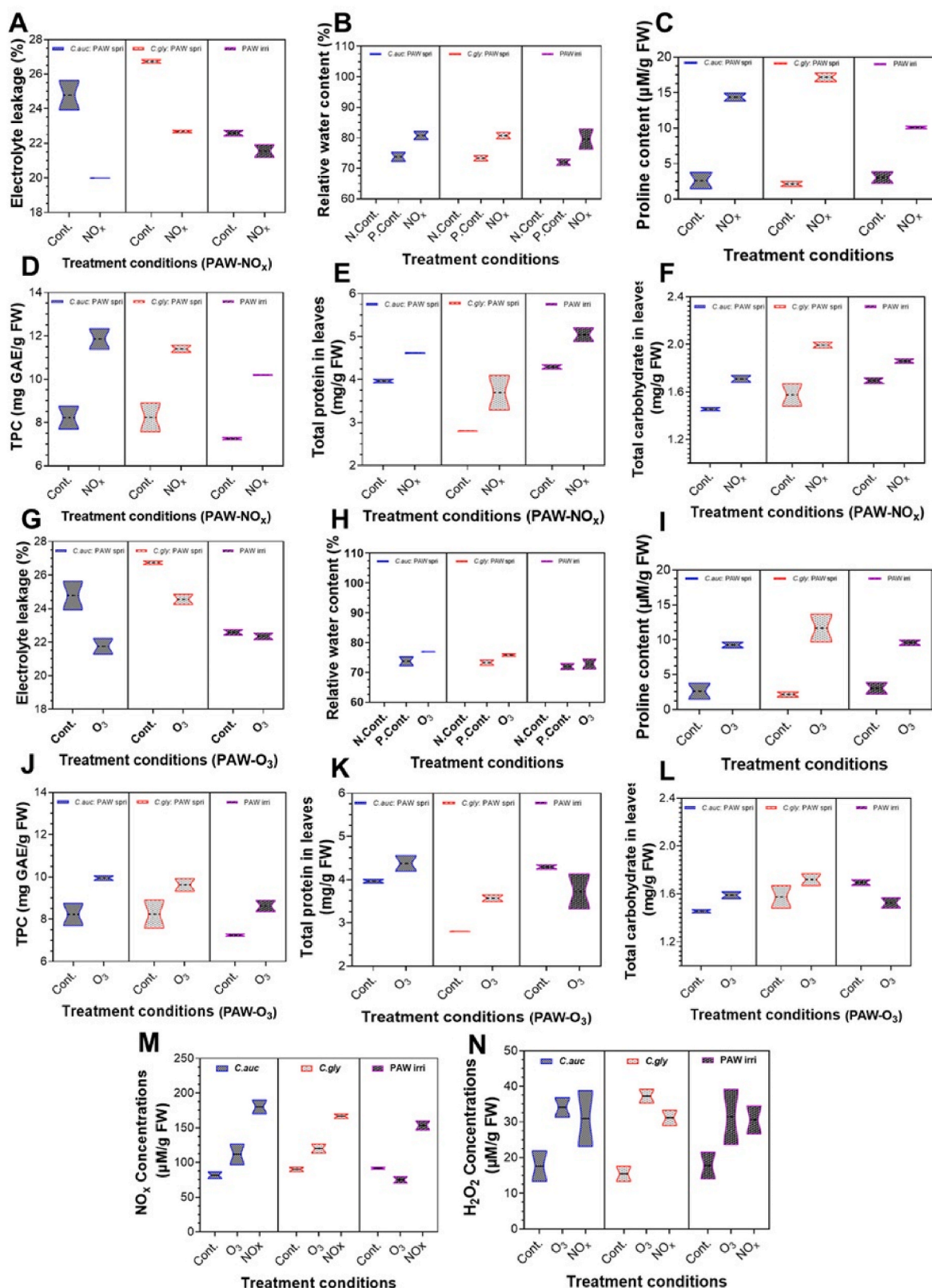


Fig. 5. Biochemical composition of strawberry leaves after fungal inactivation using two different PAW (PAW-NO_x and PAW-O₃) sprinkling and irrigation: Composition of different compounds (A) electrolytic leakage (B) relative water content, (C) proline, (D) total phenolic content (TPC), (E) protein, (F) carbohydrate were measured after PAW-NO_x spraying and irrigation. Biochemical composition after PAW-O₃ spraying and irrigation (G) electrolytic leakage, and (H) relative water content (I) proline, (J) TPC, (K) protein, (L) carbohydrate, (M) NO_x and (N) H₂O₂.

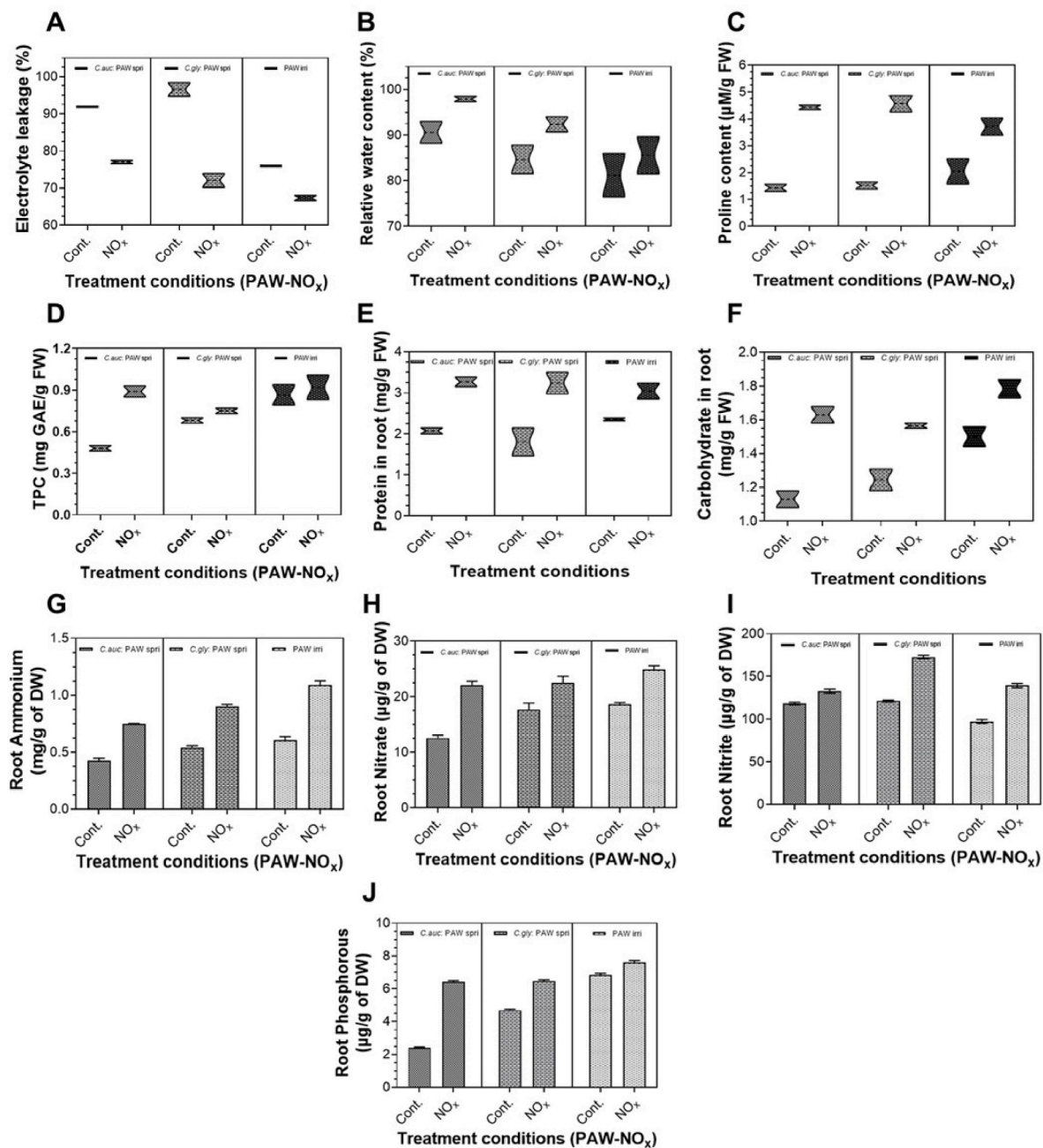


Fig. 6. Biochemical composition of strawberry root after fungal inactivation using PAW-NO_x sprinkling and irrigation: Composition of different compounds (A) electrolytic leakage, (B) relative water content, (C) proline, (D) total phenolic content (TPC), (E) protein, (F) carbohydrate, (G) ammonium, (H) nitrate, (I) nitrite, and (J) phosphorous.

same time, the carbohydrate concentrations in leaves were 1.71 ± 0.03 – 2.0 ± 0.03 , 1.53 ± 0.05 – 1.72 ± 0.06 , and 1.46 ± 0.02 – 1.70 ± 0.03 mg/g FW following the PAW-NO_x, PAW-O₃, and control, respectively (Fig. 5F–L). The concentration of NO_x during plasma treatment was as follows, 153.35 ± 7.68 – 180.00 ± 11.55 in PAW-NO_x, 75.00 ± 5.77 – 111.70 ± 17.32 in PAW-O₃, and 81.70 ± 5.77 – 91.65 ± 1.91 μM/g FW in control, respectively, with PAW-NO_x treatment significantly increasing the concentration of NO_x and decreasing it in other treatments (Fig. 5M). The PAW-O₃ irrigation reduced the NO_x concentration (75 ± 5.77 μM/g FW) compared to control because PAW-O₃ induced a necrotic effect in the strawberry plants. As shown in Fig. 5N, H₂O₂ production in leaves by spraying PAW-O₃ is higher at 37.26 ± 2.27 μM/g FW, while other PAW-O₃ irrigations are lower (30.59 ± 4.53 μM/g FW) and some PAW-NO_x irrigation increases the concentration

compared to PAW-O₃, because PAW-O₃ produced a toxic effect on the plant.

3.7. Biochemical characteristics of strawberry roots under PAW-NO_x spraying/irrigation

The EL level in PAW-NO_x treatment was 67.32 ± 0.86 – 77.03 ± 0.54 %, and in control was 75.96 ± 0.24 – 96.53 ± 2.14 % (Fig. 6A). EL was significantly decreased compared to the control. The content of RWC was significantly decreased by 81.22 ± 5.52 – 90.60 ± 2.81 % during infection and increased by 85.61 ± 4.74 – 97.92 ± 0.70 % compared to the control after PAW-NO_x treatment (Fig. 6B). The proline content in the PAW-NO_x treatment was 3.72 ± 0.38 – 4.57 ± 0.35 μM/g FW and in the control, it was 1.44 ± 0.16 – 2.05 ± 0.55 μM/g FW (Fig. 6C), and the

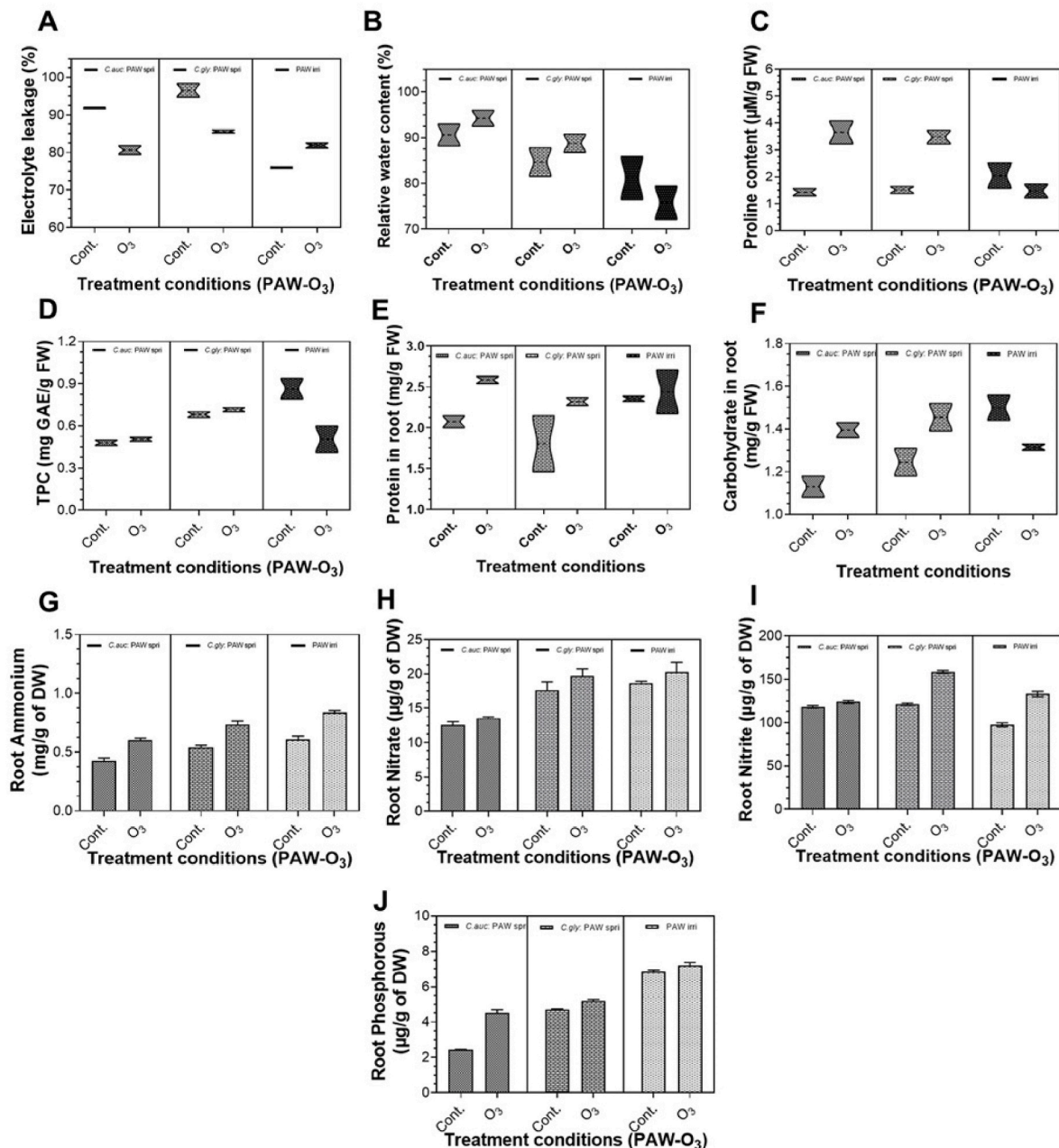


Fig. 7. Biochemical composition of strawberry root after fungal inactivation using PAW-O₃ sprinkling and irrigation: (A) Electrolytic leakage, (B) relative water content (C) proline, (D) total phenolic content (TPC), (E) protein, (F) carbohydrate, (G) ammonium, (H) nitrate, (I) nitrite, and (J) phosphorous.

amount of TPC in the PAW-NO_x treatment was 0.75 ± 0.02 – 0.92 ± 0.10 and in the control was 0.48 ± 0.02 – 0.87 ± 0.09 (mg GAE/g FW) (Fig. 6D). In these two (proline and TPC) PAW-NO_x treatments significantly increased compared to control. Soluble protein was measured in dry root after PAW spraying and irrigation. The protein content was 1.81 ± 0.40 – 2.36 ± 0.04 and 3.05 ± 0.23 – 3.27 ± 0.14 mg/g FW in the control and PAW-NO_x treatments, respectively (Fig. 6E). However, carbohydrate content was 1.13 ± 0.06 – 1.50 ± 0.07 and 1.57 ± 0.02 – 1.79 ± 0.06 mg/g FW in the control and PAW-NO_x treatments, respectively (Fig. 6F). Fig. 6G shows, the concentration of ammonium ions in roots after fungal inactivation by PAW-NO_x spraying and irrigation. PAW-NO_x has an ammonium concentration in PAW-NO_x is 0.75 ± 0.01 – 1.09 ± 0.03 and 0.42 ± 0.02 – 0.61 ± 0.03 mg/g in control respectively, and irrigation has a higher concentration of ammonium compared to control. The reason for this high ammonium ion level is the assimilation of nitrogen, where plants reduce NO₃⁻ and NO₂⁻ to

ammonium ions through a sequential process (Mahmood et al., 2023; Mokhele et al., 2012). Low level of nitrate concentrations in control (12.56 ± 0.46 – 18.68 ± 0.23 μg/g DW) and higher in PAW-NO_x (22.09 ± 0.65 – 24.90 ± 0.62 μg/g DW) (Fig. 6H). Similar patterns reflect the nitrite in both treatments; the nitrite concentration is 132.67 ± 2.25 – 172.67 ± 1.86 in PAW-NO_x and 97.37 ± 2.08 – 118.33 ± 1.37 μg/g in control respectively (Fig. 6I). Different reactive species, especially NO₃⁻ and NO₂⁻ present in the PAW after treatment, are absorbed by the plant during the sprinkle or irrigation of PAW which may have increased the amount of NO₃⁻ and NO₂⁻ in roots (Dahal et al., 2024a). Phosphorous concentration in the root is 2.42 ± 0.03 – 6.83 ± 0.09 in control and 6.43 ± 0.06 – 7.60 ± 0.10 μg/g in PAW-NO_x treatment (Fig. 6J). Between treatments, PAW irrigation contained higher levels of nutrients, and PAW treatment contained higher levels of nutrients compared to without PAW. Various reactive species present in PAW-NO_x may improve the phosphorus solubility in soil allowing plants to absorb it

more effectively as a result the phosphorus content in roots after treatment is increased (Mukhtar et al., 2020).

3.8. Biochemical characteristics of strawberry roots under PAW-O₃ sprinkling/irrigation

The EL content during infection is 91.88 ± 0.20 – 96.53 ± 2.14 % and without infection is 75.96 ± 0.24 %, after PAW-O₃ treatment reduce the EL is from 80.66 ± 1.35 – 85.62 ± 0.50 % in PAW-O₃ sprinkling and 81.84 ± 0.80 % in irrigation. In both conditions treatment significantly reduce the EL compared to control (Fig. 7A). However, during the infection period, the RWC value in control was 90.60 ± 2.81 – 84.70 ± 3.62 % by sprinkling and 81.22 ± 5.52 % by irrigation, and in PAW-O₃ treatment it was 94.27 ± 2.07 , 88.80 ± 2.31 % increased and 75.77 ± 4.24 % decreased by staking. PAW-O₃ sprinkling maintain the RWC compared to irrigation, while PAW irrigation induces a toxic effect on roots (Fig. 7B). The proline content in the control group after infection was 1.44 ± 0.16 and 1.52 ± 0.15 µg/g FW in spray and 2.05 ± 0.55 in irrigation. After PAW-O₃ treatment it was 3.66 ± 0.50 and 3.48 ± 0.30 µg/g FW in spray and 1.48 ± 0.30 µg/g FW in irrigation (Fig. 7C). Similar pattern is expressed in, TPC, protein and carbohydrate. In TPC content in the control is 0.48 ± 0.02 – 0.87 ± 0.09 and treatment is 0.51 ± 0.02 – 0.72 ± 0.02 (mg GAE/g FW), while in protein concentration in the control group is 1.81 ± 0.40 – 2.36 ± 0.04 mg/g FW and PAW-O₃ treatment 2.32 ± 0.06 – 2.59 ± 0.05 mg/g FW and also carbohydrate concentration in the control group is from 1.13 ± 0.06 – 1.50 ± 0.07 mg/g FW and PAW-O₃ treatment 1.32 ± 0.02 – 1.46 ± 0.08 mg/g FW (Fig. 7D–F). In Fig. 7B–F all the irrigation experiment indicated in control group higher the concentration compares to PAW-O₃ treatment, this may happen due to toxic effect to plant due to continuous PAW-O₃ irrigation. The ammonium ion levels in *C. acutatum* and *C. gloeosporioides* infected plants during TW spray and TW irrigation were 0.42 ± 0.02 , 0.54 ± 0.02 , and 0.61 ± 0.03 mg/g, respectively. These concentrations were slightly increased to 0.60 ± 0.01 , 0.73 ± 0.03 , and 0.84 ± 0.01 mg/g, respectively, during PAW-O₃ sprinkling and irrigation. Irrigation obtained higher levels of ammonium (Fig. 7G). Fig. 7H and I shows the NO₃[−] and NO₂[−] content in roots treated with PAW-O₃. The NO₃[−] concentration in the control was 12.56 ± 0.46 – 18.68 ± 0.23 µg/g DW and in the PAW-O₃ treatment, it was increased to 13.57 ± 0.13 – 20.22 ± 1.32 . The higher NO₃[−] was achieved in PAW-O₃ irrigation than in other sprays. Additionally, the concentration of NO₂[−] in control was found to be 118.33 ± 1.37 , 121.33 ± 1.03 , and 97.37 ± 2.08 µg/g, which was 124.00 ± 1.55 , $158.67 \pm$ and 133.00 ± 2.68 in the PAW-O₃ sprinkling and irrigation (Fig. 7I). The increase in NO₃[−] and NO₂[−] is less in this case compared to PAW-NO_x, because the formation of reactive species is less in PAW-O₃. Furthermore, the phosphorus content in PAW sprinkling after the *C. acutatum* infection increased from 2.42 ± 0.03 µg/g to 4.51 ± 0.16 µg/g after treatment. Similarly, the phosphorus concentration in *C. gloeosporioides* PAW spray increased from 4.68 ± 0.05 to 5.18 ± 0.09 µg/g and in PAW irrigation from 6.83 ± 0.09 to 7.19 ± 0.16 µg/g. The low content of reactive organics species in PAW-O₃ makes less phosphorus in the soil available to the roots (Fig. 7J).

4. Discussion

Various microorganisms are infecting or damage the strawberry plant and fruits, apart from reduce the yield and indirectly induced the environmental pollution. Pesticides are used in the agricultural sector to reduce crop losses caused by insects, many of which are harmful to fruits and human health. Other than chemicals/fungicide (Muñoz, 2002), gamma-rays and X-rays are used to control the fungus. Plasma radiation is relatively safer than ionizing radiation such as gamma rays and X-rays because the amount of energy it produces is lower than others. In food various fungi (*Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Phomopsis*, *Colletotrichum*, *Ascochyta*, *Chaetomium*, and *Rhizoctonia*) infect the strawberry and efficiently inactivate the spores in suspension

by plasma. *C. acutatum* and *C. gloeosporioides* these two fungus species significantly inhibit the growth of strawberry plants and fruits. This fungus is controlled by various methods, particularly chemicals, this chemical slightly alters the biochemical composition of the strawberry fruits. Misra et al., 2014 explained that various cold plasma methods are used to clean pesticide residues. Plasma treatment significantly inhibits the growth of the fungus in the leaves, roots, and normally existing fungus in the fruits without any side effects. According to a Adhikari et al. (2020), review by non-thermal atmospheric plasma has demonstrated its potential as an alternative tool for the effective and ecologically harmless control of plant pathogenic microorganisms for inoculation.

Fungus inactivation was attained at two different methods, 1) plasma gas exposure (NO_x gas and O₃ gas) and PAW (PAW-NO_x and PAW-O₃). Direct plasma gas (both NO_x and O₃ gas) exposure resulted in 99.99 % inactivation of *C. acutatum* within 20 min and increased *C. gly* inactivation to 99.9 % in 7 min (Fig. S2). Furthermore, NO_x and O₃ gases were used to evaluate fungal inactivation and avoid nutrient degradation in strawberry fruits without reducing their nutritional value. In both cases, a treatment time of 16 min was found to be optimal time to inactivate the fungus and NO_x gas treatment maintain the nutrients in the fruit compared to O₃ gas treatment (Figs. S 2–7). Gaseous state of the nitrogen monoxide (NO) and hydroxyl (OH) in gaseous state contain molecules dissolved into the water to forming nitrous acid, HNO₂, which may dissolve providing NO₂[−] ion in water and also leftover OH form hydrogen peroxide H₂O₂. In O₃ concentration in the gaseous stage is high, if this O₃ was reacted with water to forming hydrogen dioxide (HO₂) Eq. (29). Besides these reactive species can either decrease or increase pH. Plasma gas exposed in the water can contact with an electric current, an increase of ions (H⁺, OH[−], HNO₃, NO₂[−]), and other exciting species there in the water (Fig. 1). This plasma activation considerably changes the ionic concentration in the water and this lets to alters its electrical conductivity (Shen, Tian, Li, Ma, Zhang, et al., 2016). Oxidation-reduction potential (ORP), during plasma gas (ions, electrons, radicals, and UV radiation) exposure to tap water alters the chemical properties of the water particularly RONS (OH, O₃, NO₃[−], NO₂[−], ONOO[−]). These species induced higher oxidation-reduction potentials, due to their capability to act as an oxidant or reductant (Lukes et al., 2012). Reactive species produced by the plasma gas interact with minerals, and organic and inorganic compounds in the tap water to produce TDS, and we measured these TDS. Plasma-activated water does not have a normal salinity like that found in seawater or saline solutions. Plasma-generated RONS interact with tap water, which already contains Na, Cl, Ca, and Mg in common with tap water, thereby increasing salinity. Plasma dissociates H₂O molecules into hydrogen ions (H⁺) and hydroxide ions (OH[−]). This process, which involves reactive oxygen species (O₂ and H₂O₂), indirectly contributes to the dissolved oxygen content.

PAW significantly reduced fungi based on NO_x concentration at 48 h (Fig. 2), PAW-NO_x killed *C. acutatum* (reduced from 4.43 to 1.68 CFU/mL) by 84.5, 94.0, 98.1, 99.5, and 99.8 % of control, 50, 150, and 250 µM of NO_x and NaOCl, respectively. At 48 h, PAW-NO_x inhibited *C. gloeosporioides* (from 3.1 to 0 CFU/mL) to 98.8, 99.5, 99.9, and 99.99 % of control at 50, 150, and 250 µM NO_x concentrations and achieved 99.99 % efficiency in NaOCl for 20 h. NO_x in PAW-O₃ inhibited the growth of *C. acutatum* (reduced from 4.43 to 2.12 CFU/mL) to 79.8, 89.0, and 94.1 % of control, 15, and 30 µM of NO_x respectively and reached 99.5 % in NaOCl. Furthermore, *C. gloeosporioides* reached 99.0, 99.3 and 99.8 % in control, 15 and 30 µM of NO_x, respectively, in addition to 99.99 % in NaOCl. RONS generated from different plasma sources inactivates fungi. Using PAW-containing RONS (NO₂:80 ppm; H₂O₂: 20 ppm and O₃:0.5 ppm) generated by plasma jet inactivated *C. gloeosporioides* with a 1.5 log reduction in 30 min (Wu et al., 2019). Another study showed that gliding arc discharge significantly delayed *C. gloeosporioides* disease development in mango, with mangoes maintained at 30 °C for up to 12 days after 7 min of PAW treatment, resulting in a 1 log reduction (Phan et al., 2019). Plasma not only inactivation of

Colletotrichum sp and other species also, double-jet nozzle generated PAW contain OH inactivate less than 1 log order in *A. niger* in strawberry fruits in 7.5 min and also reduced the Aflatoxins from 223.3 to 138.9 ng/g (Ouf et al., 2016). Even though Hojnik et al. (2019) reported surface barrier discharge (SBD) generated RONS (Nitrite 150 μ M, nitrate 1000 μ M, H_2O_2 800 μ M) reduce the *A. flavus* spore within 4 min. O_3 is a strong oxidant that reacts with water to produce $OH^\bullet$, H_2O_2 , and O_2 . The O_3 half-life for aqueous ozone is usually less than 1 h due to its high reactivity (Agmon et al., 2018). Shen, Tian, Li, Ma, Zhang, et al. (2016), reported that NO_x (nitrate and nitrite) concentrations remained for up to 30 days. NO_x species such as NO_3^- and NO_2^- are long-lived species, with half-lives of many days, and O_3 has less fungal inactivation compared to NO_x due to the stability of these reactive species. The cell wall of fungi contains polysaccharides and proteins. These layers are disturbed by chemical reactions such as RONS, and this oxidation causes the fungal polyunsaturated fatty acids (i.e., lipid peroxidation) to be destroyed. This damages the vesicles and releases lipid fragments, ultimately leading to loss of plasma membrane integrity (Agmon et al., 2018). This effect occurs through the Fenton reaction (Braughler et al., 1986). This is because NO_x remains in PAW for a longer period of time, making it more effective in inactivating fungi compared to O_3 .

PAW significantly controls the fungus, reducing the fungal load on strawberries and significantly damaging biochemical compounds in 8-min treatments (immersion of strawberry fruits with and without PAW and PC). Furthermore, PAW treatment significantly maintained biochemical compositions compared to control and PC between treatments (Figs. 3 and 4). In all the treatments PAW- NO_x conditions significantly maintain or higher the following nutrients and antioxidants like nitrite 2.32 ± 0.02 mg/kg of FW, nitrate 114.53 ± 6.27 mg/kg of FW and phosphorous 54.13 ± 1.44 mg/kg of FW, protein 1.11 ± 0.07 (mg/g FW), carbohydrate 1.53 ± 0.06 (mg/g FW), proline 12.12 ± 0.76 μ M/g FW, TPC 5.62 ± 0.16 mg GAE/g FW in strawberry fruits. The biochemical composition is maintained in the following treatment regimes, which are PAW- NO_x , PAW- O_3 , PC (2000x), and control, respectively (Fig. 3H–N). *In-vivo* inactivation of *C. acutatum* and *C. gloeosporioides* on strawberry plants in open field significantly reduced the fungus with and without PAW (PAW- NO_x and PAW- O_3) treatment (spraying and irrigation). Biochemical levels were also maintained

compared to the control (Figs. 5–7). PAW- NO_x treatment was superior compared to PAW- O_3 .

Plants distinguish a biotic and abiotic modification in the environment, subsequently, plants are aerobic organisms during the biochemical process they gather and utilize molecular oxygen (O_2). In this study, PAW- O_3 irrigation induced cell death in the strawberry plant, and this may happen in high levels of ROS. During the stress time, the plants are generated a toxic byproduct entitled ROS which includes superoxide anion (O_2^-), singlet oxygen (1O_2), hydroxyl radical ($OH^\bullet$), and H_2O_2 (Choudhury et al., 2017; Mittler, 2002). Fungus is likely to reduce the levels of essential nutrients (NH_4^+ , NO_2^- , NO_3^- and phosphorus) in strawberry roots. However, the application of PAW- NO_x and PAW- O_3 through sprinkling or irrigation can effectively enhance the level of nutrients present in roots of strawberry plants. Among all these treatments, PAW- NO_x irrigation resulted in the highest nutrient concentrations, making it the most effective case. PAW- O_3 irrigation causes necrotic effects on leaves and roots, and reduces chlorophyll and nutrient contents compared to PAW- NO_x treatment. Higher levels of ROS can cause damage to proteins, carbohydrates nucleic acids, etc. ROS provokes cell death by immense redox homeostasis if oxidative stress is severe (Bartos, 1997). If the damage cannot be reversed, programmed cell death might be initiated Mittler (2002).

Several studies have demonstrated the effectiveness of using non-thermal atmospheric plasma particularly MC-DBDP to inactivate pathogenic virus (Ashokkumar et al., 2025) fungi and bacteria in fruits and plants (Fig. 8). Plasma promotes many advantages and minimizes disadvantages compared to traditional control tools like chemicals. Furthermore, plasma can successfully eliminate chemo-resistant pathogens and non-resistant pathogens because it lacks selectivity among pathogen types. Plasma gas and PAW-containing RONS (pictured) entered strawberry fruits and inactivated the fungus, while direct plasma gas (NO_x and O_3) exposed to the fruit surface to high concentrations of RONS, whose high ROS concentrations induce a necrotic effect on the fruits. Additionally, strawberry fruits/plants were soaked in PAW- NO_x , PAW- O_3 and PC for treatment. Of these, PAW- NO_x gave the best results, significantly improving the nutritional content of the fruit. These findings highlight the potential of PAW- NO_x as an effective method to improve nutrient concentration in both the fruits and roots of

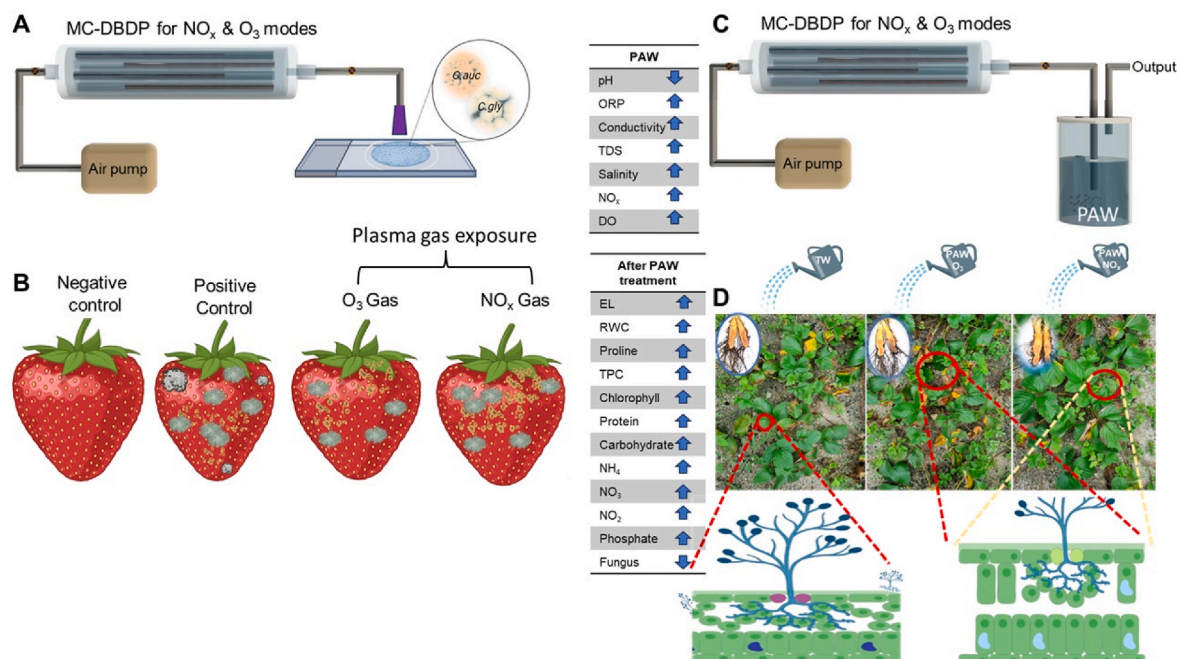


Fig. 8. Inactivation mechanisms of *Colletotrichum* sp. by plasma gas and PAW application *in-vitro* and *in-vivo*.

strawberry plants and reduce the negative effects of fungal infections. When fungal infections occur, the fungi promote cell damage (chloroplast) in the leaf and reduce water transport from one cell to another, a phenomenon that leads to water loss, electrolysis leaves behind leachate, reducing chlorophyll and other nutrients (NO_2 , NO_3 , ammonium and phosphate). Plasma-generated RNS enhances all of the above content compared to the control (Fig. 8).

5. Conclusion and future prospects

The concentration of reactive species in the NO_x and O_3 gas generated by the plasma increases with increasing treatment time, which reduces the number of fungi in various conditions (*in-vitro* and *in-vivo*). These reactive species significantly control the *Colletotrichum* sp spores on fruits/plants *in vitro* and *in vivo*, which may be due to the enrichment of reacting organisms in the limited monitoring of strawberries. These results suggest that a treatment time of 16 min is optimal for fungus inactivation and maintains the nutrient values in strawberry fruits for both NO_x and O_3 gas exposure, with a significant effect in the case of NO_x . These NO_x treatments help preserve strawberry fruits for a longer period after harvest, maintaining the freshness of strawberry fruits, while also helping to control fungus and maintain nutrient content. Sprinkling and irrigation treatments of PAW- NO_x and PAW- O_3 to inactivate fungi in strawberry plants in the fields, PAW- NO_x significantly improved plant growth and other biochemical compounds compared to PAW- O_3 and control. A sprinkling of PAW- O_3 is better compared to control, but it is not good in irrigation because PAW- O_3 induces a toxic effect (necrotic effect) on plants. Based on all the findings NO_x is better compared to other treatments. In the future, when planning large-scale use of plasma in large-scale production in the agricultural sector, we need to consider the chemical nature and toxicity of PAW, soil, and microbial conditions. We also need to fill those gaps in understanding the molecular mechanisms underlying fungal interactions in plants and fruits with reactive species present in the plasma.

CRediT authorship contribution statement

Sekar Ashokkumar: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roshani Dahal:** Methodology, Investigation, Formal analysis. **Yung Oh Shin:** Validation, Methodology, Investigation. **Han Sup Uhm:** Validation, Methodology. **Jang Sick Park:** Methodology, Investigation. **Young-Chul Lee:** Validation, Methodology, Investigation, Conceptualization. **Eun Ha Choi:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no competing financial interest or personal relationships that may have influenced the work reported in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2025.111722>.

Data availability

Data will be made available on request.

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