PEA SEED SPROUTING USING PLASMA-ACTIVATED WATER AND ASSESSMENT OF QUALITY PARAMETERS

by

SRI SAI JAYA CHANDRA GONE

(Under the Direction of Rakesh K. Singh)

ABSTRACT

This study investigates the effects of plasma-activated water (PAW) on the sprouting of pea seeds (*Pisum sativum* L.), comparing its physicochemical properties with those of potable water (PW). PW was treated with atmospheric cold plasma using two feed gases for different durations and volumes. Key physicochemical properties, including pH, electrical conductivity (EC), oxidation-reduction potential (ORP), and nitrate (NO_3^-), were monitored over 30 days. Results showed that longer plasma treatment and smaller water volumes increased EC and NO_3^- levels while pH decreased and remained stable over 30 days. Germination rates significantly improved, with seeds treated for 4 min exhibiting a 75% increase ($p<0.05$). Although total phenolic and flavonoid content was unchanged, Gamma-aminobutyric acid (GABA) levels increased slightly ($p>0.05$). Scanning electron microscopy revealed that PAW treatment effectively removed the seed's waxy surface, enhancing sprouting. These findings suggest that PAW can improve sprouting with the optimization of treatment conditions for different seed types.

INDEX WORDS: Atmospheric cold plasma (ACP), Plasma-activated water (PAW), Physicochemical properties, GABA, Seed sprouting, Storage stability.

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DEDICATION

I dedicate this work to my lovely family—Uppalaiah Gone, Balamani Gone, and Pradeep Chandra Gone—whose unwavering love, sacrifices, and trust in me shaped my journey. This work stands as a testament to your sacrifices, constant love, and enduring support.

This thesis serves as a powerful reminder of my growth and the journey I have undertaken. It reflects how far I have come and the challenges I have overcome. With this foundation, I am ready to shape my future in meaningful ways, embracing new opportunities and striving to make a positive impact.

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CHAPTER 1

INTRODUCTION

Recent trends in food consumption reflect a growing awareness of health and nutrition, with an increasing number of people incorporating seed sprouts into their daily diets. Plant sprouts from seeds, such as mung beans, alfalfa, peas, broccoli, and radish, are gaining popularity due to their high nutritional value and functional properties (Aloo et al., 2021; Baenas et al., 2017; Gabriel et al., 2007; Gan et al., 2017; Kim et al., 2017). The sprouting process enhances the availability of nutrients and reduces anti-nutritional compounds, making sprouts a valuable addition to a healthy diet (Liu et al., 2022; Mir et al., 2021).

In this study, pea seeds (*Pisum sativum* L.) were selected to investigate the effects of Plasma activated water (PAW) on seed germination and sprouting. Pea seeds are a rich source of various nutrients, including proteins, carbohydrates, minerals, and vitamins, making them a valuable subject for agricultural research (De Almeida Costa et al., 2006; Rathore et al., 2022). Despite their nutritional importance, limited studies have been conducted on the effects of PAW, specifically on pea seeds, highlighting the further need for investigation in this area.

Furthermore, pea seeds play a crucial role in advancing sustainable agriculture by reducing reliance on synthetic nitrogen fertilizers. This is facilitated by their ability to establish a symbiotic relationship with nitrogen-fixing bacteria, such as rhizobia, which facilitates biological nitrogen fixation in the soil (Abeysingha et al., 2024). Understanding the impact of PAW on pea seeds could provide insights into enhancing their growth and nutrient content, thereby contributing to more sustainable agricultural practices.

Plasma, often referred to as the fourth state of matter, has shown promise in addressing these challenges. Plasma consists of various reactive components, including ions, electrons, radicals, and UV photons (Fridman, 2008), and is classified into thermal and non-thermal (Bogaerts et al., 2002). Among non-thermal technologies, atmospheric cold plasma (ACP) has emerged as a novel approach for microbial inactivation, enzymatic treatment, and enhancing seed germination (Guragain et al., 2021; Thirumdas et al., 2015). However, direct plasma treatment may cause adverse effects, including surface topography alterations and bioactive compound

reductions (Misra et al., 2014). Additionally, the complex surface topography of food items prevents uniform plasma treatment, leaving certain regions untreated.

To overcome these challenges, PAW has been proposed as an alternative for food disinfection and plant growth enhancement (Guo et al., 2017; Xiang et al., 2019; Zhang et al., 2016; Zhang et al., 2017). PAW, enriched with reactive oxygen and nitrogen species (RONS), has demonstrated potential in bacterial inactivation and improving seed germination, with the added benefit of being easy to apply and environmentally friendly. Several studies have highlighted the ability of PAW to influence seed germination and plant growth (Billah et al., 2020; Lo Porto et al., 2018; Sivachandiran & Khacef, 2017). Furthermore, the NO_3^- content in PAW has been shown to enhance plant growth, potentially serving as a natural nutrient alternative to chemical fertilizers. However, further research is needed to fully understand the underlying mechanisms and assess the feasibility of PAW for larger-scale industrial applications.

Although PAW has a wide range of potential applications, there remains a significant gap in the literature, particularly concerning its use at larger volumes, which are essential for industrial applications. While some studies have investigated the effects of PAW on pea seed germination, the impact of PAW on bioactive compounds in pea seeds remains underexplored. The aim of this study was to address these gaps by examining the effects of varying water volumes on PAW, specifically utilizing a 500 mL volume. Pea seeds (*Pisum sativum* L.) were selected as the model for this research due to their high nutritional value, making them an ideal subject for studying the potential benefits of PAW in enhancing seed germination and bioactive compound formation. Specifically, this research aims to:

1. Characterize the physicochemical properties of PAW and assess its storage stability.
2. Evaluate the effects of PAW on sprouting time, seed germination and sprouting, and bioactive compounds of pea sprouts, including antioxidant capacity and total phenolic and flavonoid content.
3. Investigate the influence of PAW on the surface morphology and wettability properties of pea seeds.

CHAPTER 2

LITERATURE REVIEW

2.1 Sprouts

The production of sprouts and microgreens has gained significant interest across various disciplines due to their rapid growth cycle, which ranges from a few days (for sprouts) to approximately two weeks (for microgreens). Sprouting is a simple, cost-effective method that does not require sophisticated equipment, making it easily accessible. This presents a unique opportunity for industrial scalability while providing consumers access to nutrient-rich foods (Galieni et al., 2020; Renna et al., 2017). Compared to their mature counterparts, sprouts and microgreens have lower levels of anti-nutrients and higher concentrations of amino acids, fatty acids, and simple sugars, resulting from the enzymatic breakdown of larger macromolecules and bioactive compounds, known as phytochemicals (Miyahira et al., 2021).

As consumer preferences shift towards healthier diets rich in fruits and vegetables containing high amounts of bioactive compounds, the demand for ready-to-eat products has increased. Increasing the consumption of natural foods can reduce reliance on chemical additives and promote environmental sustainability. In this regard, sprouted legumes and cereals present promising options for food products with high levels of phenolic and antioxidant compounds, vitamins, and minerals. The term "sprouted seeds" encompasses various products depending on the parts consumed and the conditions under which they are grown. Definitions can vary, leading to ambiguity in terms such as microgreens and shoots. According to Regulation (EC) No 208/2013, "sprouts" are defined as the product obtained from germinated seeds harvested before true leaves develop. The American Association of Cereal Chemists (AACC), endorsed by the USDA, defines "sprouted grains" as containing all original components of the grain, provided sprouting does not exceed kernel length (Benincasa et al., 2019).

Sprouting seeds have long radiation, particularly in Eastern cultures, where sprouts are considered a vital component of culinary traditions. Since the 1980s, the popularity of sprouted seeds has surged in Western countries, driven by consumer interest in nutrition and exotic, healthy

foods. Recently, the trend has shifted towards minimally processed and additive-free options. Due to their distinctive qualities—such as vibrant colors, rich flavors, and high levels of bioactive compounds—sprouted seeds can enhance the sensory appeal of salads and serve as garnishes in various dishes. Sprouting is a straightforward and cost-effective process requiring no specialized equipment, has a rapid production cycle of two to three weeks, occupies minimal space in greenhouse settings, and yields relatively high outputs.

Additionally, the germination process does not require sunlight or soil and can occur regardless of seasonal constraints. When seeds are soaked, their digestibility increases, making sprouted beans and grains easier to digest while retaining their full micronutrients (Montemurro et al., 2019). The first step in sprouting is water absorption; during this respiration period, grains activate and produce endogenous enzymes that facilitate germination (Miyahira et al., 2021). Germination triggers significant biochemical changes in whole seeds, where the action of α-amylase mobilizes starch reserves by breaking down the granule surface and creating pinholes. Meanwhile, nitrogen-containing compounds shift towards oligopeptides and free amino acids, altering the amino acid profile. Triacylglycerols start to hydrolyze, leading to an increased ratio of saturated to unsaturated fatty acids. Additionally, the levels of anti-nutritional factors, such as phytates, trypsin inhibitors, and tannins, significantly decrease while the concentration of bioactive compounds, such as phenolics, phytosterols, folates, and GABA, increases. As a result, sprouted grains provide enhanced nutrient availability and higher levels of various antioxidants, reinforcing their classification as "functional foods."

Phytochemicals play key roles in plant defense against environmental stresses, including protection against abiotic and biotic stresses that may threaten seedling health. Certain stress conditions during germination can act as elicitors, enhancing the phytochemical content of sprouts. Excessive stress can hinder sprout growth, and careful calibration of elicitor levels is needed to optimize phytochemical yield. Increased stress levels can boost phytochemical production but may also hinder sprout growth. Thus, the application of elicitors must be carefully adjusted to determine the optimal dose that maximizes phytochemical yield, which is defined as the combination of sprout biomass and phytochemical concentration (Benincasa et al., 2019). To enhance germination rates and phytochemical content, various methods—ranging from physical and chemical techniques to combinations of both—have been explored over the past decade. These methods include gamma irradiation, electrolyzed oxidizing water, ultrasound, cold plasma, and the use of

pesticides. However, some of these technologies have been associated with adverse effects. In response to the growing demand for safety and freshness, research is increasingly focused on innovative and safe processes that improve the quality and safety of sprouts. Current investigations aim to optimize germination processes using PAW while minimizing chemical interventions economically, thereby ensuring the production of safe, high-quality sprouts.

2.2 Non-thermal cold plasma

Plasma is commonly known as the fourth state of matter. It is the ionization of neutral gases consisting of ions, free radicals, charged particles, and other molecules (Wang et al., 2022). This ionization occurs when neutral gases are energized under varying temperature and pressure conditions. Crookes made the initial discovery in 1879, but it was not until almost 50 years later, in 1929, that Tonks and Langmuir established a theoretical relationship between the arc current (independent variable) and five dependent variables: axial electric field, ionization density, electron temperature, positive ion current to the wall, and the rate of positive ion generation. This theoretical breakthrough paved the way for practical developments in plasma-generating devices (Ozen & Singh, 2020).

Plasma can be categorized into two main types based on thermodynamic properties and energy particle composition: thermal and non-thermal, with distinctions such as local thermal equilibrium. Furthermore, plasma is divided into high-temperature plasma (HTP) and low-temperature plasma (LTP) (Mildaziene et al., 2022). LTP can be classified into thermal plasma (TP), generated at temperatures exceeding 20,000 K, and cold plasma (CP) or non-thermal plasma (NTP), produced at atmospheric temperatures ranging from 30°C to 60°C. All species (electrons, ions, and neutral particles) in thermal plasma exist in thermodynamic equilibrium. In contrast, non-thermal plasma features a significantly higher electron temperature (Te) compared to the gas temperature (Tg) of heavy particles (Te >> Tg) (Bárdos & Baránková, 2010; Kogelschatz, 2003; Ozen & Singh, 2020)

Additionally, cold plasma is classified into atmospheric and low-pressure plasma based on pressure conditions. ACP can be produced without expensive reaction chambers or pumps, as it functions effectively under atmospheric conditions. This characteristic has gained increased attention in the food industry, as it does not require high temperature and pressure adjustments (Chizoba Ekezie et al., 2017; Misra et al., 2016). In this context, "cold plasma" refers to non-thermal equilibrium plasma.

Plasma can be generated using various energy sources capable of ionizing gases, including electrical, thermal, optical (UV light), and radioactive (gamma and X-ray) radiation. However, electric and electromagnetic fields are predominantly used for generating cold plasma (CP) (Pankaj et al., 2018). The supplied energy disassociates the gas into a range of reactive species, facilitating additional reactions such as excitation, de-excitation, and ionization.

Several atmospheric pressure plasma generation technologies are employed in the food industry, including dielectric barrier discharges (DBD) (Kogelschatz, 2003), corona discharges (CD) (Chang et al., 1991), microwaves (Leins et al., 2014), glow discharge plasma (GDP) (Montie et al., 2000), atmospheric pressure plasma jets (APPJ) (Ehlbeck et al., 2011), and radiofrequency discharges. DBD and APPJ are commonly used in food industries due to their ease of adoption, simple construction, and commercial availability, making them suitable for use in atmospheric conditions.

The DBD device consists of two metal electrodes, one typically coated with a dielectric barrier to prevent arc transition. This generates numerous micro-discharges for uniform treatment. When the electrodes are separated by distances ranging from 100 mm to several centimeters, an alternating current induces the formation of DBD plasma (Bárdos & Baránková, 2010; Pankaj et al., 2018).

In contrast, the APPJ device consists of two concentric electrodes. This high-frequency power ionizes the gas, typically helium, oxygen, or gas mixtures, producing a jet-like plasma appearance as the ionized gas exits through a nozzle. The high voltage (100-250 V) applied to the inner electrode ionizes the carrier gases, while the gas flow helps propel the stream of active particles away from the electrodes (Scholtz et al., 2015). When plasma is applied to liquid, it will result in plasma-activated liquid.

2.3 Plasma-activated water: Chemistry and production of PAW

PAW technology is emerging in agriculture and food science for its potential to enhance decontamination, promote plant growth, and improve food safety. Generated by atmospheric plasma discharges in water, PAW involves interactions between plasma's reactive species and water, creating a complex mixture of reactive oxygen and nitrogen species (ROS and RNS) with significant biocidal properties. PAW is generally generated in three categories: discharges of the gas phase above the liquid surface, discharges as bubbles inside the liquids, and multiphase discharges. The schematic representation of different discharge structures is shown in Figure. 2.1

by Xiang et al. (2022). Each method presents distinct advantages for PAW production, contributing to its effectiveness in different applications. PAW's chemical composition and reaction products generated above the liquid surface differ significantly from those produced within the liquid. When plasma is discharged below the water surface, the water itself becomes part of the discharge process, resulting in more intense reactions and a greater production of reactive species. The type and concentration of these reactive species are influenced by the specific gases and liquids used in the PAW generation process.

Additionally, factors such as the type of plasma device, plasma activation time, the feed gas, input voltage, storage time, the type of liquid used, and the volume of the liquid and the chemical environment all play a role in producing the PAW (Han et al., 2023; Lim et al., 2021; Liu et al., 2020; Lukes et al., 2014; Lukeš et al., 2012; Ma et al., 2015; Vaka et al., 2019). The distance between the liquid and the plasma plume plays a crucial role in forming PAW. When oxygen, nitrogen, and water are used as the main components, a range of primary reactive species is generated, including hydroxyl radicals, atomic oxygen, atomic nitrogen, superoxide, and nitric oxide. These primary species include singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroxyl radical ($\cdot OH$), peroxynitrite ($ONOO^-$), and nitric oxide ($NO\cdot$). When these reactive species interact with the liquid, they give rise to various long-lasting species, including nitrite (NO_2^-), nitrate (NO_3^-), and hydrogen peroxide (H_2O_2). Understanding this dynamic is essential for optimizing PAW production and enhancing its efficacy in various applications (Brisset & Pawlat, 2016; Lukeš et al., 2012). When plasma interacts with water, it produces PAW. Various complex reactions occur at the gas-liquid interface region, creating an acidic condition and forming reactive oxygen species (ROS) and reactive nitrogen species (RNS). This further leads to various primary and secondary reactive species dissolved in the liquid and changes in PAW's redox potential and conductivity (Gorbanev et al., 2018; Xiang et al., 2022). These species alter the physicochemical characteristics, lowering the pH and increasing its EC and ORP. When these effects are combined with ROS and RNS, they work together synergistically to enhance the decontamination activity of PAW (Thirumdas et al., 2018). Table 2.1 outlines the chemical reactions during PAW generation, and Table 2.2 summarizes the reactive species generated at different stages of PAW (Han et al., 2023). A deeper understanding of the chemical reactions involving these species, alongside the physicochemical characteristics of PAW, will facilitate investigating its potential applications.

2.4 PAW generation techniques

Various Plasma sources, such as DBD, GAD, and SMD, generate PAW. However, the DBD and plasma jet are popular in the food industry because of their versatility and simple and adaptive working use (Pankaj et al., 2014). Apart from the plasma sources, the treatment time and gases used, the distance between the liquid and plasma plume, and the nature of electrodes are important issues that should be considered. The operating parameters can also significantly affect the concentration of reactive species. These parameters will include the type of feeding gases, the gas flow rate, the volume of treated water, electrical power, and treatment duration.

2.5 Feed gases

The type of carrier gas used to generate atmospheric pressure plasma significantly influences the concentration of reactive species in the plasma and the physicochemical properties of the PAW (Thirumdas et al., 2018; Zhou et al., 2019). Commonly utilized gases include nitrogen, oxygen, noble gases such as argon and helium, and various gas mixtures. Air emerged as the most frequently used gas due to its economic and logistical advantages (Chen et al., 2019; Ma et al., 2016; Zhou et al., 2019). The reactive species formed using different gases are shown in Figure. 2.2.

The duration of plasma treatment also affects the physicochemical properties of PAW, which varies according to the type of gas employed. Helium and oxygen discharges generally produce more alkaline pH levels, while nitrogen and air discharges result in acidic activated water due to the reaction of reactive nitrogen oxides with water. The RNS in PAW originated from dissolved nitrogen compounds, with higher RNS content observed when air or nitrogen served as the feed gas. In contrast, using oxygen or argon promoted the formation of ROS, such as H_2O_2 (Guo et al., 2021).

Zhou et al. (2016) investigated the effects of different feed gases (O_2, N_2, He, and air) on the physicochemical properties of PAW. They found that air-activated PAW exhibited the highest ORP of 650 mV, the lowest pH of 3.5, and the highest concentrations of long-lived ROS and RNS. Lai et al. (2020) further examined gas combinations, concluding that nitrogen-containing mixtures led to increased acidity due to $NO\cdot$ dissolution while adding oxygen resulted in a moderate pH decrease. Maximum concentrations of NO_3^-, NO_2^-, and H_2O_2 were observed with various gas mixtures, including [Ar + air/Ar], [Ar + air/air], and [Ar/Ar], respectively. Nitrogen-containing

mixtures favored nitrate formation, whereas air promoted nitrite production, with pure argon enhancing H_2O_2 generation.

Another study explored the physicochemical properties of PAW using varying N_2/O_2 ratios as carrier gases, finding a strong correlation between reactive species in PAW and the gas composition. The highest H_2O_2 concentration was achieved at a N_2/O_2 ratio of 80/20, resembling an air-like mixture. As O_2 content increased, the number of oxygen atoms rose, leading to greater ozone (O_3) production. Nitrate concentrations increased from 81.6 to 166.4 mg/L as the N_2/O_2 ratio shifted from 100/0 to 50/50 before decreasing with further O_2 increments. Notably, nitrite levels were negligible when pure nitrogen was used as the carrier gas. Before plasma treatment, the pH, EC, and ORP values of deionized water (DW) were 7.3, 1.18 μS/cm, and 394.42 mV, respectively. After plasma activation at a N_2/O_2 ratio 50/50, the pH decreased significantly, while EC and ORP values increased to 2.2, 706 μS/cm, and 550 mV, respectively. However, further increases in O_2 proportion led to decreases in conductivity and ORP.

Shainsky et al. (2012) investigated the pH dependence of PAW generated from various feed gases (Ar, O_2, air). They found that PAW produced from argon did not significantly alter water pH, while PAW generated from oxygen and air exhibited drops in pH to 2.01 and 2.07, respectively. Bolouki et al. (2021) also explored the effects of different feed gases (air, nitrogen, oxygen, and argon) on PAW. They reported no significant changes in pH with oxygen and argon plasmas. In contrast, PAW from air and nitrogen plasmas showed a significant decrease in pH over time, attributed to limited nitrogen interaction and ambient air during treatment with oxygen and argon, resulting in minimal nitrite and nitrate levels. Their study indicated that PAW generated by oxygen plasma had the highest levels of H_2O_2, with pH values between six and eight.

In conclusion, the choice of carrier gas in atmospheric pressure plasma generation plays a critical role in determining the physicochemical properties of PAW, particularly regarding pH, ORP, and the concentrations of reactive species. All the above studies indicate that gases such as air and nitrogen result in more acidic PAW due to the formation of reactive nitrogen species, while noble gases like argon or oxygen contribute to higher levels of reactive oxygen species and a less acidic environment. Especially air has been shown to yield PAW with significant concentrations of both reactive oxygen and nitrogen species, maximizing ORP and enhancing the overall reactivity of the resulting solution. Varying gas composition and treatment duration highlight the

need to choose suitable gas mixtures for specific applications of PAW relevant to areas ranging from agriculture to healthcare.

In addition to carrier gas type, gas flow rate influences the PAW properties. Lower flow rates allowed the working gas to remain in the plasma discharge region longer, increasing plasma density. Conversely, higher gas flow rates generate greater plasma output per unit of time (Guo et al., 2021). Uchida et al. (2015) determined that emission behaviors near the water interface were notably impacted by gas velocity, with increased gas velocity expanding the plasma irradiation area on the water surface and facilitating a greater influx of charged particles into the water medium. Similarly, Hou et al. (2021) a study conducted to assess the physicochemical properties of PAW was treated at airflow rates of 6 and 10 (SLM) for 20 min. The pH of PAW dropped significantly to a range of 2.59 and 2.89, respectively, from an initial pH of around 7.0, with no notable differences in pH, ORP, and EC values between the two flow rates. While ozone concentrations remained constant, H_2O_2 concentrations decreased significantly when a higher flow rate was used. NO_3^- and NO_2^- concentrations in PAW significantly increased with higher plasma flow rates ($p < 0.005$). In another study by Chuea-uan et al. (2024) found that treating deionized water for 15 min at various flow rates (2, 3, 4, 5, and 6 L/min) resulted in a significant decrease in pH as flow rates increased, with notable increases in NO_3^- content at 5 and 6 L/min compared to lower rates.

Similarly, significant increases in NO_2^- and H_2O_2 concentrations were observed across flow rates, although no significant differences were noted in ORP or EC. Another study by Naeim and Humud (2023) investigated distilled water treated with argon gas at varying flow rates (0.7, 1, 1.5, and 2.1 L/min). Compared to the other treatments, they identified 0.7 L/min as the optimal flow rate for maximizing reactive oxygen and nitrogen species (RONS) production.

In addition to the gas composition and flow rate, the volume of liquid used to generate PAW significantly influences the concentration of its physiochemical species. The concentration of reactive species increases as the sample volume decreases and the plasma treatment time increases. A study by Rathore and Nema (2021) found that increasing the discharge power of a pencil plasma jet in ultrapure water led to higher EC and ORP in PAW while the pH decreased. Conversely, increasing water volume at constant power raised the pH but lowered ORP and EC values. Kutasi et al. (2019) examined the physiochemical properties of PAW over different treatment volumes (25 mL, 125 mL, and 200 mL) and found that H_2O_2 concentrations increased

linearly, regardless of volume. However, NO_3^- and NO_2^- concentrations saturated at the lower 25 mL volume due to higher H_2O_2 levels, a trend not seen in larger volumes.

Furthermore, pH decreased linearly with treatment time, aligning with increases in nitrite and nitrate concentrations. The most significant initial decrease in concentration occurred in the 25 mL volume, where NO_2^- quickly declined due to recombination with H_2O_2. The lower H_2O_2 concentrations in larger volumes resulted in more moderate recombination, allowing NO_2^- levels to remain stable for months (Kutasi et al., 2019).

2.6 Storage time

The efficiency of plasma-activated liquid depends on various factors, including plasma system and processing parameters, exposure time, and post-generation parameters, such as storage temperature and duration. As time elapses, the characteristics of the PAW will change. One of the main advantages of using PAW against non-thermal gas plasma is its storability. Extended storage duration will impact the levels of the PAW's physicochemical properties. For instance, Zhao et al. (2020) used atmospheric cold plasma to produce PAW by treating water for 5 min. They found that after 48 h of storage, H_2O_2 concentrations significantly decreased, nitrite levels declined, and nitrate concentrations increased. The pH and ORP showed minimal changes over 24 h at 4°C, though electrical conductivity increased.

Similarly, Andreev et al. (2019) prepared PAW using electrode-microwave discharge plasma, reporting an H_2O_2 content of 3×10^{-3} mol L^{-1} that remained stable for over seven days. Vlad and Anghel (2017) observed that H_2O_2 levels remained stable for extended periods, lasting up to seven days and beyond 21 days. This stability suggests the potential for producing PAW with longer shelf lives.

In contrast, NO_2^- concentrations varied with temperature, remaining stable at -80 °C while declining at higher temperatures. Specifically, nitrite levels decreased from 1.2 μM at 25 °C to 0.1 μM, 0.4 μM, and 0.6 μM at 4 °C, −20 °C, and -80 °C, respectively, over the 30 days. While PAW stored at -80 °C exhibited significantly higher concentrations of H_2O_2 and nitrite compared to other temperatures, indicating that this storage condition may enhance PAW's effectiveness for sterilization. While pH, ORP, and nitrate levels showed minimal variation, increased storage temperatures correlated with reduced concentrations of H_2O_2 and NO_3^-. The interactions among nitrites, H_2O_2, and temperature will likely influence PAW's biological activity, highlighting the importance of optimizing storage conditions to preserve its efficacy. A study examined the

stability of PAL, including PAW and plasma-activated saline, over 18 months at varying temperatures: room temperature (20 ºC), 4 °C, -16 °C, -80 °C, and -150 °C. Throughout this duration, pH levels remained stable and unaffected by ambient and sub-ambient temperatures. ROS measurements indicated an initial decrease after one month for all PAL, followed by a rebound at three months and a subsequent decline at 18 months. This pattern suggests that ROS in PAL may continue to react with other species, such as RNS. Notably, PAW stored at -80 °C and -150 °C after one month exhibited ROS concentrations comparable to those of PAW stored at -16°C after six months. Additionally, nitrite levels were undetectable in all samples, and no notable differences in nitrate levels were found across the various storage conditions (Tsoukou et al., 2020).

In a study by Wang and Salvi (2021), the stability of PAW was analyzed at two temperatures (22 ± 1°C and 4 ± 1°C) over 15 min. The pH of PAW remained stable at both temperatures. ORP increased at 4 °C, while EC positively influenced stability over 4 to 72 h at 4 ± 1°C. Nitrite levels decreased significantly over time, with better performance at 4 ± 1°C during the 16–72-h period. Lower temperatures did not enhance nitrate stability, and freezing and thawing did not significantly alter PAW's properties or its effectiveness against E. coli; PAW from frozen samples showed slightly greater effectiveness than the control group (Wang & Salvi, 2021). In another study by Chuea-uan et al. (2024), GA plasma was discharged above DI water and stored for 20 days. The resulting PAW was analyzed immediately and stored at room temperature (20 ºC) for 20 days. Throughout the 20 days, levels of NO_3^-, NO_2^-, and H_2O_2 continued to decrease, while pH, EC, and ORP remained stable.

In conclusion, storage temperature and duration factors significantly influence the stability and efficacy of PAL. While physicochemical properties like pH and ORP remain relatively stable, concentrations of reactive species, including H_2O_2, nitrite, and nitrate, vary over time and conditions. Optimal storage conditions, particularly low temperatures, enhance the effectiveness of PAW for sterilization. Future research should focus on refining these storage parameters to maximize the therapeutic potential of PAL.

2.7 Activation time

Activation time refers to the duration of plasma contact with water, significantly influencing RONS production. An increase in activation time results in a greater interaction between plasma and water, thereby generating higher concentrations of RONS (Lietz & Kushner,

2016). For instance, Laurita et al. (2015) demonstrated that longer treatment durations led to elevated nitrate and H_2O_2 levels. However, it is important to note that nitrite in PAW exhibited instability in acidic environments (pH < 3.5), showing a decreasing trend with extended treatment time (Lukes et al., 2014). In a study conducted by Fan et al. (2020), after being activated by plasma for 90 s, the concentration of NO_2^-, NO_3^-, and H_2O_2 in PAW increased ($p<0.05$), respectively. The pH value of PAW significantly decreased with increasing discharge time, and ORP and EC increased. The water acidification is mainly due to the formation of nitrous, nitric, and peroxynitrite acid inside the liquid during the plasma activation (Sivachandiran & Khacef, 2017; Thirumdas et al., 2018). Similarly, Ma et al. (2015) reported that EC, temperature, and ORP increased with longer plasma activation times, while pH decreased initially and stabilized around 3 after 20 min. It is also supported by other research, which reported similar results but did not detect the NO_2^- ions in all the treatments (Than et al., 2022).

Kučerová et al. (2019) found that plasma-activated tap water (PATW) maintained a relatively stable pH of 7.5, attributed to a natural hydrocarbon buffer system, with only mild pH changes during activation. Similarly, Park et al. (2013) demonstrated that in transfer arc treatments, the pH initially increased but gradually decreased with longer activation times. This relationship between activation time and pH changes highlights the importance of optimizing treatment duration for specific applications in food and agriculture.

2.8 PAW generation method

The physicochemical properties of PAW vary significantly based on its generation method, categorized into two pathways: above the water surface (PAW-A) and beneath the water surface (PAW-B). In PAW-A, plasma radiation alters water composition at the gas-water interface. Mass transfer is crucial here, but barriers can limit the interaction between gas and water. Enhancing the reactor design to improve gas-water mixing can help overcome these limitations. Conversely, in PAW-B, RONS are generated in the gas phase, allowing only long-lived species to dissolve into the solution. This method leads to a more intense reaction and a greater production of active species. Additionally, in direct contact modes, the distance between the plasma source and the water surface significantly affects the generation of reactive species through plasma-liquid interactions, further influencing the overall composition of PAW. A study Uchida et al. (2015) demonstrated that the production of RONS in PAW can be selectively modulated by adjusting this distance, allowing for control over the NO_2 to H_2O_2 concentration ratio across a broad range.

Similarly, Kaushik et al. (2018) examined how varying distances (1 to 4 mm) influenced the production of N_2 and OH species near the water surface. The results showed that with increasing distance, the concentration of OH decreased while electron density increased. This suggests that precise distance adjustments can customize RONS production, highlighting the importance of reactor design in optimizing PAW characteristics for various applications. Lu et al. (2017) conducted comparative studies on the generation of reactive species in water exposed to open-air spark discharge (SD) and glow discharge (GD) plasmas, revealing significant differences in the composition of PAW. Their findings indicated that SD-PAW contained both H_2O_2 and NO_3^-, while GD-PAW primarily featured NO_2^- and NO_3^-. Notably, the total concentration of reactive species was higher in SD-PAW than in GD-PAW. In another comparative study Tsoukou et al. (2021) utilizing a similar plasma unit, both types of PAWS exhibited a drop in pH from neutral to acidic levels. After 5 min of plasma exposure, the pH reached 3.03 for SD-PAW and 2.7 for GD-PAW, remaining stable for a week post-treatment. The conductivity of both SD and GD PAW increased significantly and remained stable after one week. H_2O_2 was detectable exclusively in SD PAW, with concentrations stable over time, while nitrites were found only in GD PAW and declined after one week. Nitrate concentrations rose with plasma activation time in SD and GD PAW, with no significant changes noted after a week.

In a study by Medvecka et al. (2022), the properties of PAW produced by high-voltage pulsed corona discharge (CD) and high-power atmospheric plasma jets (APJ) were examined. This research compared the properties of PAW generated at the gas plasma/water interface with those produced by underwater discharge within the same system, emphasizing the critical role of nitrogen in facilitating the formation of reactive nitrogen species (RNS) in PAW. The results indicated that PAW generated above the water surface exhibited significant changes in properties compared to that produced underwater, including a decrease in pH, an increase in ORP, a marked rise in conductivity, and the formation of RNS. Additionally, Tian et al. (2015) found that PAW generated beneath the water surface (PAW-B) displayed higher ORP, greater conductivity, and stronger disinfection efficacy than PAW produced above the water surface (PAW-A). These findings highlight that the generation method of PAW significantly affects its physicochemical properties, with PAW-A and PAW-B exhibiting distinct characteristics and reactivity.

2.9 Application of PAW on seed germination or sprouting

Germination is a cost-effective and straightforward method for seed growth. The process involves a triphasic water uptake pattern in mature, dry seeds: rapid imbibition (phase I), a lag phase (phase II), and a subsequent increase in water absorption (phase III) (Nguyen et al., 2017). Unlike plants, which can harness energy from their environment, grain and legume seeds depend on breaking down stored compounds to generate the energy needed for growth. Enzymatic hydrolysis is crucial in the catabolic process, converting storage macromolecules like carbohydrates, proteins, and fats into easily usable forms. Moreover, hydrolysis helps reduce antinutrients like phytic acid and tannins, enhancing the nutritional value of the seeds.

PAW has emerged as a valuable resource in the agriculture sector, particularly for enhancing seed germination and sprout production. While numerous studies have reported positive effects of PAW on seed germination, growth, and reduction of the sprouting time, these findings remain inconsistent due to the complex nature of plasma treatment (Fan et al., 2020; Ji et al., 2022; Lo Porto et al., 2018; Zhou et al., 2019). While some studies have shown a positive effect of PAW on germination, others have shown no effects (Guragain et al., 2021; Liu et al., 2019; Wang et al., 2022). To understand the primary mechanism involved in dormancy and germination, two hormones primarily regulate the process: abscisic acid (ABA) and gibberellic acid (GA). ABA promotes dormancy and inhibits germination, while GA promotes germination and counters ABA's effects. The balance of these hormones can be influenced by external factors, particularly reactive species like H_2O_2, nitrates, and nitric oxide.

H_2O_2 plays a dual role in seed physiology and stress response. It is a signaling molecule that regulates ABA and GA metabolism (Liu et al., 2010). It can disrupt dormancy by enhancing ABA breakdown and promoting GA synthesis, often mediated by nitric oxide (Bahin et al., 2011). Additionally, high levels of ABA can inhibit GA production, with H_2O_2 activating GA signaling instead of altering ABA metabolism.

The effects of PAW on seed germination can be divided into three main aspects. Firstly, the ROS produced by PAW can help crack the seed coat, improving water and nutrient absorption. This increases germination, and the vigor index and H_2O_2 within the ROS can help break down hard seed coats, allowing for greater moisture absorption (Chen et al., 1993).

Secondly, the reactive nitrogen species (RNS) present in PAW are essential nutrients for seed germination. Nitrate is a nutrient that aids in producing amino acids and nitrogen compounds.

It is also a signaling molecule that influences plant development (Wang et al., 2003). Seeds can absorb nitrate, which is then converted into nitrite by nitrate reductase and further reduced by nitrite reductase to form ammonium ions. These ammonium ions are critical components in the synthesis of amino acids (Duermeyer et al., 2018). Finally, RONS, including H_2O_2 and nitrate, act as signaling molecules that stimulate seed germination. They trigger proteomic, transcriptomic, and hormonal changes, promoting germination (Barba-Espín et al., 2012). Understanding these mechanisms is vital for optimizing PAW applications in agricultural practices. This suggests that ROS like H_2O_2, dissolved O_3, etc., may be involved in damaging the hydrophobic wax on the surface of seeds, which allows water to be absorbed quickly.

Additionally, PAW contains reactive nitrogen species (RNS) in the form of NO_2^- and NO_3^- ions, making it a rich nitrogen source. PAW is a nutrient under certain conditions, such as germination and initial growth stages. Nitrogen, the building block of biomolecules such as amino acids, plays a crucial role in this process (Rathore et al., 2022).

Previous studies have demonstrated that PAW effectively reduces germination time and enhances seed germination rates, with processing conditions such as power, current, frequency, voltage, gas flow rate, and sample size crucial for understanding PAW's influence on seeds. Research by (Sivachandiran & Khacef, 2017) on radish (*Raphanus sativus*), tomato (*Solanum lycopersicum*), and sweet pepper (*Capsicum annuum*) using DBD plasma indicated that nitrates and nitrites in PAW function as fertilizers, promoting both seed germination and seedling growth. However, longer exposure of seeds and water to plasma has been associated with negative effects, as noted in other studies.

Zhou et al. (2019) demonstrated that PAW generated by atmospheric pressure plasma significantly enhanced the germination and growth of mung bean sprouts. This improvement is attributed to various RONS present in PAW, which may activate the plant's defense mechanisms. Billah et al. (2020) also investigated the effects of PAW treatment on black gram seeds, exposing them to PAW for durations ranging from 3 to 15 min. Their findings revealed that PAW-treated seeds exhibited higher germination rates than untreated controls, with an optimal treatment duration of 3 min yielding the highest vigor index and 6 min resulting in the best cumulative germination rate. These results highlight the beneficial effects of PAW on seed germination and growth, with outcomes varying based on treatment duration.

Conversely, Darmanin et al. (2021) investigated on mung beans, and it was found that combined treatment with direct air cold atmospheric plasma (CAP) and air PAW did not adversely affect germination. Similarly, Stoleru et al. (2020) studied *Lactuca sativa L.* in various pot volumes and found that while PAW concentrations did not affect the germination rate, they positively influenced the lengths of radicles and hypocotyls. Wang et al. (2022) reported similar outcomes in buckwheat sprouts. Additionally, Guragain et al. (2021) a positive impact was observed in radishes and peas, though fenugreek seeds showed no significant change in final germination rates.

These studies suggest that the seed type and the duration of PAW treatment must be optimized for each specific seed variety. Overall, the impact of PAW on seed germination is complex, with variations resulting from different plasma sources, plant species, treatment durations, feed gas compositions, and seed characteristics. Understanding these factors is crucial for optimizing PAW applications in agricultural practices.

2.10 Effect of PAW on bioactive compounds of sprouts

Bioactive compounds, particularly total phenols and flavonoids, play an essential role in plant defense. The primary bioactive compounds found in seeds and sprouts are flavonoids and phenols (Chon, 2013). Substantial alterations in the composition of bioactive substances within seeds accompany the dynamic germination process. These bioactive substances encompass a spectrum of essential compounds, including but not limited to gamma-aminobutyric acid (GABA), flavonoids, and polyphenols; remarkably, the transformation of these constituents undergoes a heightened degree of modification when subjected to stress conditions, with non-thermal plasma emerging as a particularly impactful stressor in this context. Under normal germination conditions, seeds exhibit intrinsic biochemical changes as they transition from dormancy to active growth. However, applying stress, such as exposure to non-thermal plasma, amplifies the magnitude of these alterations. Various studies have shown PAW's positive and negative effects on sprouts. For instance, Wang et al. (2022) examined the impact of PAW on buckwheat sprouts, observing increases in stem length, flavonoid and phenolic content, and antioxidant activity compared to controls. Similarly, the effects of PAW on mung bean sprouts subjected to plasma were studied for varying durations, noting increases in germination rates, total phenolic and flavonoid content, and antioxidant capacity. However, these effects diminished at longer activation times (30-90 s) (Fan et al., 2020).

A study conducted by Xiang et al. (2019) reported that no significant differences were observed in the TPC and TFC of sprouts treated with PAW. This is a great advantage of the plasma treatment method, as the sensitive bioactive component stays unchanged in treated samples. However, this result strongly depends on the food matrix, the treatment time, and the plasma condition, including the applied voltage.

2.11 Effect of PAW on GABA

Gamma-aminobutyric acid (GABA), a bioactive non-protein amino acid with the molecular formula $C_4H_9NO_2$ and a molecular weight of 103 Da, is primarily synthesized through the decarboxylation of L-glutamic acid by the enzyme glutamic acid decarboxylase (GAD; EC 4.1.1.15). GABA functions mainly as an inhibitory neurotransmitter, crucial in regulating neuronal excitability in the central nervous system. It plays a crucial role in regulating neuronal excitability and has been associated with various physiological benefits, including cardiovascular regulation, diabetes management, and anxiety reduction. Given its potential health benefits, there is growing interest in enhancing GABA levels in pulses rich in protein (Diana et al., 2014; Nikmaram et al., 2017).

Recent studies indicate that non-thermal plasma (NTP) treatment can significantly promote GABA synthesis in plant seeds (Chen et al., 2016; Pal et al., 2016). The reactive species generated during NTP treatment—such as radicals, ROS, and ultraviolet light—may induce stress responses in seeds that enhance GABA accumulation. Although oxidative modification of amino acids by ozone in NTP treatment has been suggested to alter amino acid composition, the mechanisms underlying GABA accumulation in NTP-treated rice flour remain unclear (Pal et al., 2016).

During germination, seeds undergo environmental stressors that can elevate GABA levels as a critical signaling molecule that regulates various metabolic and transcriptional processes (Kinnersley & Turano, 2000). For example, Chou et al. (2021) demonstrated that plasma treatment at 80 W on mung bean sprouts significantly increased GABA content, achieving levels 2.98 times higher than the control. However, the relationship between plasma power and GABA synthesis is not linear; excessive plasma generation may inhibit GABA biosynthesis or accelerate its degradation (Bor & Turkan, 2019; Nikmaram et al., 2017).

Another study by Wang et al. (2023) observed that PAW treatments enhanced GABA levels compared to control samples, attributing this effect to osmotic and oxidative stresses induced by low pH and the presence of NO_2^- and nitrate NO_3^-. Variations in GABA and proline

levels among different PAW treatments were likely due to differential stress responses. Conversely, Wang et al. (2022) reported that GABA levels in Tatary buckwheat sprouts increased with longer germination times, yet no significant differences were observed between the treated and control groups. In conclusion, the effects of NTP or PAW demonstrate distinct impacts that depend on the seed type and treatment conditions.

2.12 Effect of PAW on structural changes and morphological of pea seeds

During germination, seeds first absorb water, triggering a series of biochemical processes that activate the seed's enzyme system. This activation converts storage materials into soluble sugars, fatty acids, and amino acids, supplying vital energy and nutrients for the growing embryo. The seed radicle emerges through the seed coat as the embryo cells expand.

Studies have shown that ROS and RNS generated by PAW can etch the seed coat's surface, reducing its thickness and potentially enhancing water absorption. For example, Fan et al. (2020) found that seeds soaked in PAW90 exhibited a significantly higher water absorption ($p < 0.05$) compared to those treated with distilled water. Scanning electron microscopy (SEM) analysis revealed altered surface structures in PAW-treated seeds, suggesting that the high concentrations of RONS may contribute to the etching of the seed coat, facilitating greater water and nutrient uptake (Zhou et al., 2019).

Conversely, Rathore et al. (2022) found no significant effect of PAW on water uptake in pea seeds, reporting no change in water-holding capacity before and after treatment. However, PAW did modify the seeds' surface morphology by removing the hydrophobic wax layer that inhibits water absorption. This wax, which naturally coats seeds, delays soaking time. The RONS in PAW, including H_2O_2, dissolved O_3, NO_3^-, and NO_2^-, oxidize and facilitate the removal of this wax, enhancing the seeds' hydrophilicity. Billah et al. (2020) also used SEM to analyze black gram seeds and found that PAW treatment smoothed the irregular structures of the seed coats, indicating potential wax removal due to interactions with NO_2^- and H_2O_2. To further elucidate the effects of PAW on seeds, Chen et al. (2023) employed Fourier-transform infrared (FTIR) spectroscopy to analyze the chemical bonds on soybean seed surfaces before and after PAW treatment. The findings indicated that spectra of all treatments showed close similarities; only peaks displayed different intensities, which indicates that no new groups were formed and no chemical changes occurred after treatment with PAW. The mild nature of non-direct plasma is likely to minimize the harmful effects and damages to the sprouts compared to direct cold plasma. The results show that

PAW may be a safer alternative for sprouting compared to direct plasma treatment without significantly changing the seeds' physical and chemical properties.

2.13 Advantages and limitations

Cold plasma treatment of water through, i.e., PAW shows significant potential for enhancing germination rates and improving food safety and quality. PAW technology is low-cost and operates without limitations on usage and storage (Gao et al., 2022; Guo et al., 2021). It offers numerous advantages, including high efficiency, flexibility, environmental safety, and the absence of harmful residues. PAW is rich in nitrogen species that act as nutrients and can inhibit hormones responsible for seed dormancy, making it an effective eco-friendly disinfectant and plant seed stimulant. While existing studies indicate that PAW improves germination, they typically utilize small water volumes, highlighting the need for further research with larger sample sizes to determine industrially relevant parameters, such as processing times and gas flow rates. Air is the most cost-effective feed gas for the production of PAW, as demonstrated in various studies, providing an economical way to enhance germination while maintaining product quality (Chen et al., 2019; Ma et al., 2016; Zhou et al., 2019).

However, comprehensive evaluations of PAW's impact on food quality and safety—including its effects on germination, sprouting time, microbial inactivation, and nutritional changes are essential. The complexity of atmospheric plasma devices and variations in design and operation result in differing reaction compounds and mechanisms. This complexity complicates the control of reactions, and it is crucial to ensure that PAW-treated foods do not contain toxic residues or undergo undesirable chemical changes. Furthermore, while PAW does not alter sample temperatures and exhibits high efficiency and environmental safety, its short half-life of reactive species, such as hydroxyl radicals (OH) and singlet oxygen ($^1O_2$), poses challenges. PAW generated above the water surface shows different biological effects than those produced underwater, necessitating further exploration of its properties. Additionally, PAW's limited penetration into food products suggests that it should be used with other non-thermal technologies to enhance effectiveness. Optimizing treatment conditions is crucial, particularly since food components can reduce PAW's efficacy. Overall, ongoing research is needed to fully understand and harness the potential of PAW in agricultural and food safety applications.

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Table 2.1 Chemical reactions taking place during PAW generation resulting in the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

$H_2O + e \rightarrow OH\bullet + H\bullet + e^-$	(1)
$H_2O + e \rightarrow H^+ + OH\bullet + 2e^-$	(2)
$H_2O + e \rightarrow H\bullet + O\bullet + H\bullet + e^-$	(3)
$O_2 + e \rightarrow O^+ + O + 2e$	(4)
$O_2 + e \rightarrow O^- + O$	(5)
$O + O_2 \rightarrow O_3$	(6)
$O_3 + NO \rightarrow NO_2 + O_2$	(7)
$N + O_2 \rightarrow NO + O$	(8)
$O + N_2 \rightarrow NO + N$	(9)
$O + NO_2 \rightarrow NO + O_2$	(10)
$2NO + O_2 \rightarrow 2NO_2$	(11)
$NO_2 + OH \rightarrow HNO_3$	(12)
$H_2O_2 + h\nu \rightarrow OH\bullet + OH\bullet$	(13)
$3NO_2 + H2O \rightarrow 2HNO_3 + NO$	(14)
$H_2O_2 + H^+ + NO_2 \rightarrow ONOOH + H_2O$	(15)
$OH\bullet + OH\bullet \rightarrow H_2O_2$	(16)
$NO + NO \rightarrow N_2 + O_2$	(17)
$NO + OH\bullet \rightarrow HNO_2$	(18)
$HNO_2 + OH\bullet \rightarrow NO_2 + H_2O$	(19)
$NO_2 + h\nu \rightarrow NO + O\bullet$	(20)
$NO_3 + h\nu \rightarrow NO + O_2$	(21)
$NO_2 + NO_3 \rightarrow N_2O_5$	(22)
$N_2O_5 + H_2O \rightarrow 2HNO_3$	(23)
$2NO_2 + H_2O \rightarrow NO_2 + NO_3 + 2H^+$	(24)
$3NO_2^- + 3H^+ \rightarrow 2NO + NO3^- + H3O^+$	(25)
$OH + NO_2 \rightarrow [O=N–OOH] \rightarrow O=N–OO^- + H^+$	(26)

Table 2.2 Reactive species generated at different stages of plasma-activated water (PAW)

Different Stages	**Reactive species formed**
Gas phase	O_3, H_2O_2, HNO_2, HNO_3, NO, HO_2, OH, O, NO_2, NO_3, H, $1O_2$, N_2^+
Plasma-liquid interface	$H2O_2$, NO_2^-, NO_3^-, O_3, O_2^-, NO, OH,O, NO_3, H, NO_2, $ONOO^-$
Liquid phase	H_2O_2, NO_2^-, NO_3^-, O_3, O_2^-, $ONOO^-$

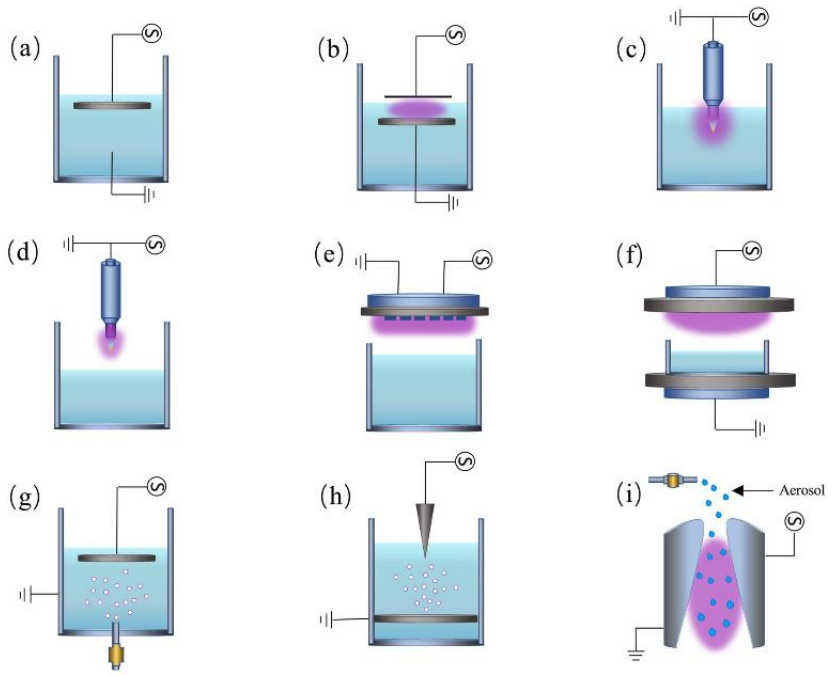

Figure. 2.1 Schematic representation of the different generations of plasma-activated water.

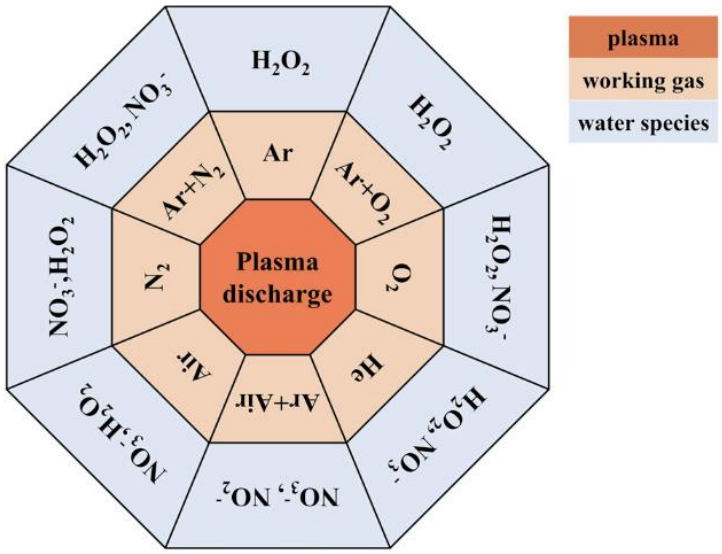

Figure. 2.2 Effect of feed gases on the formation of reactive species in water.

CHAPTER 3

Physicochemical Properties and Storage Stability of Plasma-Activated Water (PAW)[1]

[1] To be submitted to Trends in Chemical Engineering

Abstract

This study evaluated the physicochemical properties of potable water (PW) treated with atmospheric cold plasma (ACP) with two different gas compositions (80 % nitrogen + 20 % oxygen and 90 % nitrogen + 10 % oxygen). PW samples, varying volumes (30 and 500 mL), were treated for 5 and 10 min. The stability of the resulting PAW's physicochemical properties (pH, EC, ORP, NO_3^-, NO_2^-, and O_3) was assessed at room temperature (22 ± 2 °C) over a 30-day storage period. The results showed that increasing the plasma treatment time and reducing the water volume led to higher values for all measurements except pH. The results also showed that the volume of the water and discharge time notably influenced the physicochemical properties and activity of PAW. Specifically, pH, EC, and NO_3^- concentrations remained stable for 30 days without significant changes ($p>0.05$). Conversely, the ORP decreased significantly with the storage time. After PAW generation, O_3 concentration diminished to zero within a few hours, and the NO_2^- levels decreased to undetectable levels by the third day.

Keywords: Atmospheric cold plasma (ACP), Physicochemical properties, Plasma activated water (PAW), Storage stability.

Introduction

Plasma is defined as the fourth state of matter. It is the process of ionizing neutral gases, leading to the presence of ions, free radicals, charged particles, and other molecules (Bárdos & Baránková, 2010; Pankaj et al., 2014; Segat et al., 2016; Wang et al., 2022; Xiang et al., 2022). Atmospheric cold plasma (ACP) applications have gained significant attention in food processing to ensure food safety, modify biomolecules, and preserve food-bioactive compounds (Ganesan et al., 2021). PAW is generated by non-thermal atmospheric plasma active particles interacting with water (Ma et al., 2015; Shen et al., 2016). PAW is generally divided into two categories: discharges above the liquid surface and discharges inside the liquid (Xiang et al., 2022).

When plasma encounters water, it produces PAW, initiating various complex reactions at the gas-liquid interface. These acidic reactions form reactive oxygen and nitrogen species (RONS). This further leads to different primary and secondary reactive species dissolved in the liquid and changes in the redox potential and conductivity (Gorbanev et al., 2018; Xiang et al., 2022). During plasma discharge, the species formed in the PAW include short-lived species such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydroxyl radical (·OH), peroxynitrite ($ONOO^-$), nitric oxide (NO·). Upon interaction with the water, numerous long-lived species, such as nitrite (NO_2^-), nitrate (NO_3^-), hydrogen peroxide (H_2O_2), and ozone (O_3), are formed (Han et al., 2023), which leads to changes in the physicochemical characteristics of PAW. These changes make the water reactive, which includes a reduction in the pH and an increase in its oxidation-reduction potential (ORP) and electrical conductivity (EC). Numerous studies have reported that an increase in plasma discharge enhances the concentration of reactive species in the water. This increase in reactive species is associated with decreased pH values and increased ORP and EC (Guragain et al., 2021; Lo Porto et al., 2018; Wang et al., 2023; Xiang et al., 2019). The physical properties, such as surface tension and viscosity, also change with plasma activation (Shaji et al., 2023).

PAW offers several advantages, including enhanced mobility, reduced chemical residues, environmental friendliness, low cost, and no adverse effects on product quality (Han et al., 2023; Wang et al., 2022). Due to these potential benefits, PAW is gaining attention in the food industry for microbial disinfection, enzymatic inactivation to enhance seed germination, meat curing,

pesticide degradation, and more (Fan et al., 2020; Tan & Karwe, 2021; Vaka et al., 2019; Waghmare, 2021; Wang & Salvi, 2021). It is essential to examine the active components of PAW to fully understand its potential application.

Several factors significantly affect the quantity and types of RONS produced in PAW, including the plasma device used, the duration of plasma activation, the composition of the working gas, the input voltage, the storage time, and the volume of the liquid (Brisset & Pawlat, 2016; Liu et al., 2020; Lukes et al., 2014; Rathore & Nema, 2021; Takamatsu et al., 2014; Zhou et al., 2016). Since different conditions provide various reaction compounds, understanding the physicochemical properties was critical for standardizing PAW, as the interaction between plasma generation, plasma-liquid interactions, and biological applications is becoming increasingly important.

The literature contains a few contradictions regarding PAW's storage stability. Some studies suggest that PAW remains stable over the storage period (Andreev et al., 2019; Tsoukou et al., 2020; Vlad & Anghel, 2017). Others have observed a concentration of PAW physicochemical properties, and their effect on bacterial activity decreased over time (Shen et al., 2016; Zhang et al., 2024). Despite numerous studies, the understanding of the characterization and stabilization of PAW remains incomplete. This study explores the relationship between the concentration of reactive species, water volume, discharge time, and gas composition.

This study aims to investigate the PAW's physicochemical properties under varying conditions, including two different volumes, treatment times, and gas compositions, and evaluate its stability when stored at room temperature ($22 \pm 2\ ^{o}C$). The effect on pH, EC, ORP, H_2O_2, NO_2^-, NO_3^-, and O_3 was monitored up to 30 days after PAW generation. Understanding these physicochemical properties before and after storage is important for practical applications in food industries and agriculture.

Material and Methods

Atmospheric cold plasma

The cold plasma system (Plasma jet CD50 source, Plasmatreat GmbH, Germany) consists of a 1kV-16A power generator (FG5001) and a plasma jet (CD50) with a high-voltage transformer illustrated (Sharma & Singh, 2022). The plasma jet was generated at atmospheric pressure using high-purity (99.99%) nitrogen (N_2) and oxygen (O_2) as two different feed gases at a voltage of 2 kV with frequencies ranging from 15 to 25 Hz. The ionized gas forms a jet stream of active plasma species outside the nozzle from the ionization chamber. The flow controllers regulated the working gas, directing it into the plasma unit at a feed gas rate of 10 L/min.

In the experiment, the reactor consists of a polytetrafluoroethylene (PTFE) tube with a nozzle outlet for the gas to pass through. The design of the plasma column is shown in Figure 3.1. The bubbles formed in the water allow uniform gas mixing.

PAW generation

Figure 3.1 illustrates the generation of PAW by treating PW with a plasma jet. As per manufacturer guidelines and preliminary experiments, two different volumes of water were treated with two different gas compositions at a flow rate of 10 L/min. For measurement, 30 mL of PW was taken in a 50 mL beaker, and 500 mL was taken in a 600 mL. The water was then subjected to plasma by inserting a flow nozzle. Two different processing gas compositions were used: (i) simulated air (80% N_2 and 20% O_2) (SA) and (ii) modified air (90% N_2 and 10% O_2) (MA). The water was treated for 0, 5, and 10 min. The water generated using SA and MA treatment was named atmospheric plasma-activated water (APAW) and modified atmospheric plasma-activated water (MAPAW), respectively. The PAW produced was utilized to determine reactive species' physicochemical properties and storage stability over 30 days at room temperature (22± 2 ^oC). All the measurements were performed in triplicates at room temperature (22± 2 ^oC). The possible primary gas and liquid phase interactions (Thirumdas et al., 2018).

In this experiment, there were nine treatments named as:

Control: Potable water (PW).

A30/5: 30 mL volume treated with SA for 5 min.

A30/10: 30 mL volume treated with SA for 10 min.

A500/5: 500 mL volume treated with SA for 5 min.

A500/10: 500 mL volume treated with SA for 10 min.

M30/5: 30 mL volume treated with MA for 5 min.

M30/10: 30 mL volume treated with MA for 10 min.

M500/5: 500 mL volume treated with MA for 5 min.

M500/10: 500 mL volume treated with MA for 10 min.

Characterization of PAW's physicochemical properties

pH, Electrical Conductivity (EC), and Oxidation-reduction potential (ORP)

The pH was measured using a pH meter (Hanna edge), calibrated with pH 4, 7, and 10 buffer solutions, covering a pH range of 0-14. An ORP meter (Hanna Instruments HI98120, Romania) and a conductivity meter (Hanna HI-981303, Romania) were used to determine the ORP and EC values, respectively. Initial PAW measurements were taken immediately, and further measurements of the samples were taken throughout storage at room temperature (22 ± 2 ^{o}C).

Determination of NO_3^-, NO_2^-

The concentrations of NO_3 and NO_2 ions were determined using the colorimetric method, as reported by (Pandey et al., 2023). The calibration curve was obtained for nitrite ions using a sodium nitrite solution with a concentration ranging from 50 to 300 µg/L. The relationship between the absorbance value and NO_2^- concentration was established, as shown in Equation 1.

$$Y = 0.0004X + 0.0039 \ (R^2 = 0.999) \quad (1)$$

where Y is the absorbance of NO_2^- at 543 nm, and X is the NO_2^- concentration in µg/L.

To quantify nitrite (NO_2^-), 3 mL of plasma-activated water (PAW) was mixed with 120 µL of Griess reagent and incubated for 30 min. The absorbance was then measured at 543 nm. For nitrate ion (NO_3^-) determination, a calibration curve was constructed using sodium nitrate solutions with concentrations ranging from 1 to 5 mg/L. The relationship between the absorbance value and the concentration of NO_3^- was established, as shown in the Equation 2:

$$Y = 0.0483X + 0.0009 \ (R^2 = 0.9993) \quad (2)$$

where Y is the absorbance of NO_3^- at 220 nm, and X is the NO_3^- concentration in mg/L.

To analyze NO_3^-, 3 mL of PAW was mixed with 60 µL of 1N HCL, after which absorbance was measured at 220 nm and 275 nm.

Final absorbance = Absorbance value at 220 nm – 2* Absorbance value at 275 nm.

Determination of H_2O_2

The calibration curve uses a 30% H_2O_2 solution with concentrations ranging from 0.01 mM to 1 mM. The relationship between the absorbance value and the concentration of H_2O_2 is shown in Equation 3:

$$Y = 0.1974X - 0.0028(R^2 = 0.9993) \quad (3)$$

where Y is the absorbance of H_2O_2 at 407 nm, and X is the H_2O_2 concentration in mM.

The H_2O_2 of PAW was determined following the outlined method (Pandey et al., 2023) with slight modifications. For H_2O_2, 1.5 mL of 0.1M Titanium oxysulfate and 1.5 mL of PAW were incubated for 30 min, after which absorbance was measured at 407 nm.

Determination of ozone (O_3)

The ozone concentration in the PAW was measured immediately after processing using a commercially available ozone test kit (range: 0 to 5 ppm: vacu-vials, OzoneI-2019, Calverton, VA, USA).

Storage stability of PAW

The storage stability of PAW was assessed at room temperature (22 ± 2 ^oC). The pH, ORP, EC, NO_3^-, NO_2^-, H_2O_2, and O_3 measurements were taken on days 1, 3, 5, 7, 11, 14, and 30 to evaluate its physicochemical properties and stability.

Statistical analysis

Three replicates were taken for each measurement. Data analysis was conducted using GraphPad Prism version 10.2.2 (GraphPad Software, San Diego, CA, USA), employing analysis of variance (ANOVA) to evaluate the results. Statistical significance was established at ($p < 0.05$), with mean differences analyzed using Tukey's multiple comparison test.

Results and Discussion

Effect of plasma treatment time and volumes on the physicochemical properties of PAW

pH

The PAW's physicochemical properties were characterized by activation time, gas composition, and water volume treated. The pH of PW treated using two gas ratios (SA and MA) is illustrated in Table 3.1. The pH of PW subjected to SA and MA treatments showed a significant decrease ($p<0.05$) with increasing plasma treatment time, attributed to the higher formation of oxidizing and reactive species in the PAW. Initially, the pH of the PW was 6.92 ± 0.08. After treatments A30/5 and A30/10, the pH significantly dropped to 2.68 ± 0.20 and 2.29 ± 0.09, respectively. Similarly, for treatments M30/5 and M30/10, the pH decreased to 2.72 ± 0.03 and 2.48 ± 0.07, respectively.

The decrease in pH is mainly due to the generation of active ions, such as hydrogen ions (H^+), nitrite (NO_2^-), and nitrate (NO_3^-), during the plasma exposure (Rathore & Nema, 2021). As plasma treatment time increased, the concentration of H^+ ions in the water also increased, leading to a reduction in the pH of PAW. The interaction between plasma species and water resulted in the formation of reactive nitrogen species (RNS), including nitrous acid (HNO_2), nitric acid (HNO_3), and peroxynitrous acid (ONOOH), which contributed to the acidification process. In the case of the 500 mL sample volume, acidification was less pronounced, possibly due to insufficient formation of RNS in the larger volume of water. However, treatments with 500 mL volume, such as A500/5 and A500/10, showed slight pH reductions to 6.39 ± 0.13 and 3.95 ± 0.08, respectively. Similarly, in treatments with 500 mL and a mixture of gases (M500/5 and M500/10), pH values decreased further to 6.44 ± 0.12 and 4.30 ± 0.18, respectively.

Lower pH values were observed in treatments with smaller water volumes and longer treatment times, likely due to the higher concentration of reactive species in these samples. These results indicate that water volume significantly influences the pH of PAW. The observed acidity of PAW suggests that nitrate (NO_3^-) was the primary reactive species produced in this study.

Oxidation-Reduction Potential

ORP is a measure of the ability of a solution to oxidize or reduce another substance, indicating its ability to accept or donate an electron during a chemical reaction (Hoeben et al., 2019). A positive value of ORP indicates an oxidation potential, whereas a negative value denotes a reduction potential (Zhang et al., 2016). Additionally, ORP provides the net combination of ROS in water. Table 3.1 depicts the ORP value of PW treated with PA and MA. Before plasma treatment, the ORP value of PW was 426.33 ± 18.33 mV. Post-treatment, there was a significant increase ($P < 0.05$) in ORP across all the treatments. The ORP values of 10 min treated samples were nearly twice the ORP value of PW. The high ORP of PAW species is mainly due to the formation of various reactive chemicals, such as NO_3^-, NO_2^-, and ONOOH (Kaushik et al., 2018). The higher ORP value corresponded to a lower pH value. There is no significant difference between APAW and MAPAW treatments with ORP value. These findings are supported by previous physicochemical studies of PAW with varying plasma treatment times (Ma et al., 2015).

Electrical Conductivity

The EC measures the dissolved active ions that allow water to conduct electricity (Guragain et al., 2021; Thirumdas et al., 2018). When plasma interacts with water, it depletes the water molecules, creating H^+ and OH^- ions. Furthermore, it was found that a decrease in pH leads to an increase in EC, which may be caused by the conversion of NO_2^- to NO_3^-, NO, and H_3O^+ in PAW. PAW contains more active ions, which can be explained by the change in H+, OH^-, and NO_3^- in Equation 4 (Vlad & Anghel, 2017). These reactive species significantly contribute to the water's EC due to their ability to carry an electric charge.

$$3NO_2^- + 3H^+ \rightarrow 2NO + NO_3^- + H_3O^+ \quad (4)$$

Table 3.1 shows the EC values of PW generated with two gas mixtures (PA and MA). The initial conductivity of PW was 147.33 ± 17.01 μS/cm. Post-plasma treatment increased the EC of PAW, indicating a rise in the concentration of reactive species and ions. The plasma treatment significantly increased conductivity, peaking at approximately 2000 μS/cm for treatments A30/10 and M30/10. There was a slight increase in the treatments A500/5, A500/10, M500/5, and M500/10, but it was insignificant ($p > 0.05$). Similarly to ORP, no significant difference existed between the EC values of APAW and MAPAW treatments.

Chemical analysis of PAW

Nitrate and Nitrite:

Nitrogen and oxygen molecules dissociate into atomic N and O by electron impact; these further react and form nitrogen oxides, as shown in Equation (5-8) (Royintarat et al., 2019).

$$N_2 + e^- \rightarrow 2N + e^- \quad (5)$$

$$O_2 + e^- \rightarrow 2O + e^- \quad (6)$$

$$O + N \rightarrow NO \quad (7)$$

$$O + NO \rightarrow NO_2 \quad (8)$$

During the plasma-water interaction, the generated nitrogen dioxide dissolves in water. It then reacts with other molecules, forming RNSs, mainly NO_2^- and NO_3^-, in PAW. This process is responsible for the water's acidification, decreasing the pH (Chen et al., 2023; Royintarat et al., 2019; Zhang et al., 2024).

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (9)$$

Table 3.1 shows the NO_3^- content in PAW treated with SA and MA for different treatment times. A significant increase in NO_3^- content was observed in plasma-treated samples compared to the PW ($P < 0.05$). However, there was no significant difference between the SA and MA treatments ($p > 0.05$). The NO_3^- ion concentration showed a significant positive correlation with treatment time, although the volume of water had a greater influence on the NO_3^- content than the treatment duration. When the equal volumes were treated, no significant difference in NO_3^- content was observed with increasing discharge time. This may be due to the low discharge power and too fast flow rate, resulting in insufficient activation of the PAW (Zhang et al., 2024). The reduction in pH of PAW is partially associated with the formation of nitrogen compounds, including singlet oxygen, as shown in the above equations.

The NO_2^- content significantly increased ($p < 0.05$) with longer plasma discharge, but detectable nitrite concentrations were only observed in the 30 mL treated samples. This may be because of a lower sample volume, which allowed for more efficient interaction between nitrate and oxygen to react with water (Royintarat et al., 2019). Table 3.1 displays the NO_2^- content in PAW treated with PA and MA for various treatment times. Initially, no NO_2^- ions were detected in the PW. After treatment, the NO_2^- concentration increased, peaking at 87.68 μg/L for M500/10 treatment. Higher nitrate concentrations were observed in MAPAW samples compared to APAW samples, possibly due to the high amount of nitrogen atoms in MA. The oxidation of nitrites in PAW is driven by hydroxyl radicals (OH•) and other reactive oxygen species (ROS), including ozone (O_3). These reactive species facilitate the conversion of nitrites (NO_2^-) to nitrates (NO_3^-), thereby reducing overall reactive species within PAW, as shown in Equations 10 and 11 (Royintarat et al., 2019).

$$NO_2^- + 2OH \rightarrow NO_3^- + H_2O \quad (10)$$

$$O_3 + NO_2^- \rightarrow O_2 + NO_3^- \quad (11)$$

Hydrogen Peroxide

H_2O_2 was identified as an important and long-lived ROS species in PAW. It plays a key role as a signalling molecule in various metabolic processes and is important in biotic and abiotic stress (Zhang et al., 2017). Although PAW generally contains a measurable amount of H_2O_2, it was not detected in any of the samples analyzed in this study. This absence may be due to the high flow rate, which increased the reactivity of PAW, as indicated by low pH and high EC. This increased reactivity may have facilitated the interactions between H_2O_2, dissolved O_3, and NO_2^- ions, producing more NO_3^- ions (Zhang et al., 2024). The findings are consistent with previous studies (Vaka et al., 2019; Wang et al., 2023; Wang & Salvi, 2021).

Ozone

As shown in Table 3.1, dissolved ozone (O_3) increased with longer plasma treatment time. This may be because, during the discharge phase, O_3 can dissolve directly in the oxygen bubbles

formed in the water, leading to its accumulation in PAW as discharge time increases (Zhang et al., 2024). Initially, no dissolved O_3 was detected in the PW; however, after plasma treatment, the O_3 concentration increased, peaking at 1.32 ± 0.10 mg/L in treatment A500/10, compared to 0.79 ± 0.18 mg/L in M500/10. Additionally, O_3 concentrations were significantly higher in the APAW samples compared to MAPAW samples, with APAW samples exhibiting approximately 85% higher O_3 concentration. This is likely due to the greater presence of oxygen atoms in APAW. In addition, plasma electrons can dissociate O_2 into atoms (O), as shown in Equation 12, where M represents other molecules in the reactor, such as N_2 and O_2 (Royintarat et al., 2019).

$$O_2 + O + M \rightarrow O_3 + M \quad (12)$$

Stability study of PAW physicochemical properties

The stability of PAW storage is a major concern, particularly due to its wide range of applications in the food industry, such as disinfectants, seed germination, and microbial inactivation. However, there is a lack of comprehensive studies on the changes in PAW's physicochemical properties during post-discharge storage. To maximize the effectiveness of PAW, it is crucial to understand how its properties change over time. Therefore, this study investigated the stability and aging of PAW over a 30-day storage period at room temperature (22 ± 2 °C).

No significant difference in pH and EC were observed across all treatments throughout the 30-day storage period. As shown in Figures 3.2 (a) and 3.2 (b), the pH and EC values of PAW-treated samples remained stable during this period. However, as illustrated in Figure 3.2 (c), a significant change ($p < 0.05$) was observed in the ORP values over the 30-day storage period. These findings are consistent with the results reported by Shen et al. (2016).

As shown in Figure 3.2 (d), there was no significant change ($p < 0.05$) in the concentration of NO_3^- ions, which remained stable across all treatments. However, the NO_2^- ions did not behave similarly to the NO_3^- ions and were quite unstable, as shown in Figure 3.2 (e). The concentration of NO_2^- ions decreased rapidly, with all samples showing a decline on day 2 and the content reaching zero by day 3. This rapid decline is likely due to the conversion of NO_2^- to NO_3^- under

acidic conditions. The following chemical reaction may explain the loss of NO_2^- in PAW (Royintarat et al., 2019).

$$O_3 + NO_2^- \rightarrow O_2 + NO_3^- \quad (13)$$

Vlad and Anghel (2017) assessed the time evolution of the PAW and found that its properties have remained stable for more than 21 days. Specifically, pH, EC, and the concentrations of the H_2O_2 and nitric acid exhibited only minor fluctuations over the three-week period. Figure 3.2 (f) illustrates the ozone concentration of PAW over the storage period. The dissolved ozone concentration of samples A30/5, A30/10, A500/5, A500/10, M30/5, M30/10, M500/5, M500/10 dropped from 1.14 ± 0.06, 1.28 ± 0.06, 1.35 ± 0.05, 1.32 ± 0.10, 0.67 ± 0.16, 0.69 ± 0.04, 0.67 ± 0.16, 0.79 ± 0.18 to 0 mg/L, respectively, within an hour. This may be because of the reaction of dissolved O_3 with NO_2^- ions present in PAW, as shown in Equation 10.

Conclusions

PAW was generated using a dielectric barrier discharge device using a nitrogen and oxygen gas mixture under treatment times of 5 and 10 min and volumes of 30 mL and 500 mL. The EC, ORP, NO_3^-, and NO_2^- levels increased with treatment time, while the pH value decreased, regardless of the water volume. Notably, all these parameters were relatively higher in PAW treatment at 30 mL volume compared to 500 mL, except for pH, which showed the opposite trend. Over a 30-day storage period at 22 ± 2°C, the pH, EC, and NO_3^- concentrations remained stable and did not show significant changes, whereas the ORP decreased significantly over time in all treated samples. No H_2O_2 was detected in any of the samples. Additionally, NO_2^- ions decreased significantly and reached zero by day 3, while O_3 content dropped to zero within an hour after treatment.

These findings indicate that the physiochemical properties of PAW are influenced by factors such as water volume, gas composition, and treatment duration. The stability observed over the long storage period indicates that PAW with a desired concentration of reactive species and extended shelf life can be procured by choosing proper treatment conditions. This establishes the framework for applying PAW in the food and agriculture sector. Future research should explore additional storage conditions to further optimize PAW stability and potential uses.

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Table 3.1 Physicochemical properties of plasma-activated water with simulated air (SA; 80% nitrogen and 20% oxygen) and modified air (MA; 90% nitrogen and 10% oxygen) used as feed gases for 5 and 10 min at 30 and 500 mL water volume.

Treatments	pH	EC(µS/cm)	ORP (mV)	NO_3 (mg/L)	NO_2 (µg/L)	O_3 (PPM)
Control	6.92 ± 0.08 [a]	147.33 ± 17.01[c]	426.33 ± 18.33 [e]	4.02 ± 0.30 [e]	ND	ND
A30/5	2.68 ±0.20 [d]	1912.66 ± 78.49 [a]	558.66 ± 5.13 [d]	69.92 ± 1.41 [a]	56.13 ± 3.00 [c]	1.14 ± 0.06 [a]
A30/10	2.29 ± 0.09 [e]	≤ 2000 [a]	992.66 ± 11.01 [a]	72.17 ± 0.20 [a]	77.20 ± 6.58 [a, b]	1.28 ± 0.06 [a]
A500/5	6.39 ± 0.13 [b]	189.66 ± 65.61 [c]	884.33 ± 50.58 [c]	37.67 ± 2.24 [c]	ND	1.35 ± 0.05 [a]
A500/10	3.95 ± 0.08 [c]	213.33 ± 18.93 [c]	972.00 ± 25.53 [a, b]	47.19 ± 1.68 [b]	ND	1.32 ± 0.10 [a]
M30/5	2.72 ± 0.03 [d]	1382.66 ± 87.78 [b]	895.33 ± 51.93 [b, c]	68.56 ± 0.67 [a]	67.36 ± 3.79 [b]	0.67 ± 0.16 [b]
M30/10	2.48 ± 0.07 [d, e]	≤2000 [a]	986.00 ± 21.70 [a]	71.13 ± 0.31 [a]	87.86 ± 4.08 [a]	0.69 ± 0.04 [b]
M500/5	6.44 ± 0.12 [b]	139.33 ± 1.15 [c]	839.66 ± 25.14 [c]	20.04 ± 1.55 [d]	ND	0.67 ± 0.16 [b]
M500/10	4.30 ± 0.18 [c]	151.33 ± 7.76 [c]	990.66 ± 16.16 [c]	35.56 ± 4.97 [c]	ND	0.79 ± 0.18 [b]

Different lowercase letters indicate significant differences ($p < 0.05$). ND = not detected. EC = electrical conductivity; ORP= Oxidation reduction potential, NO_3 = Nitrate, NO_2 = Nitrite, O_3 = Ozone.

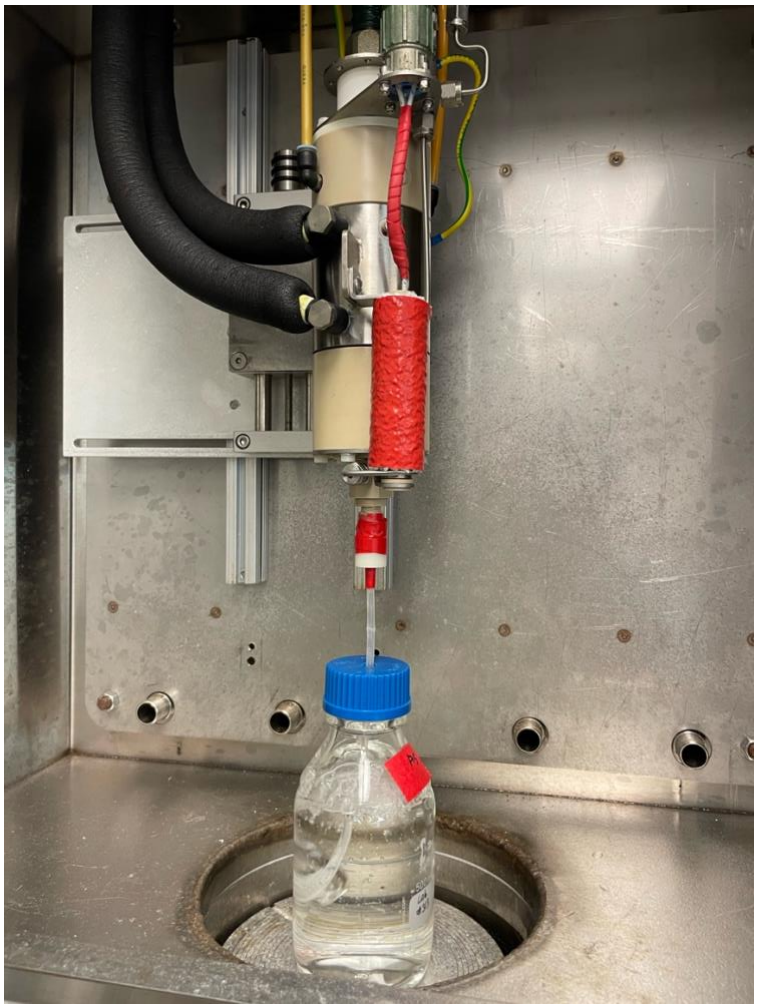

Figure. 3.1 Cold plasma treatment of potable water (PW) using the DBD plasma.

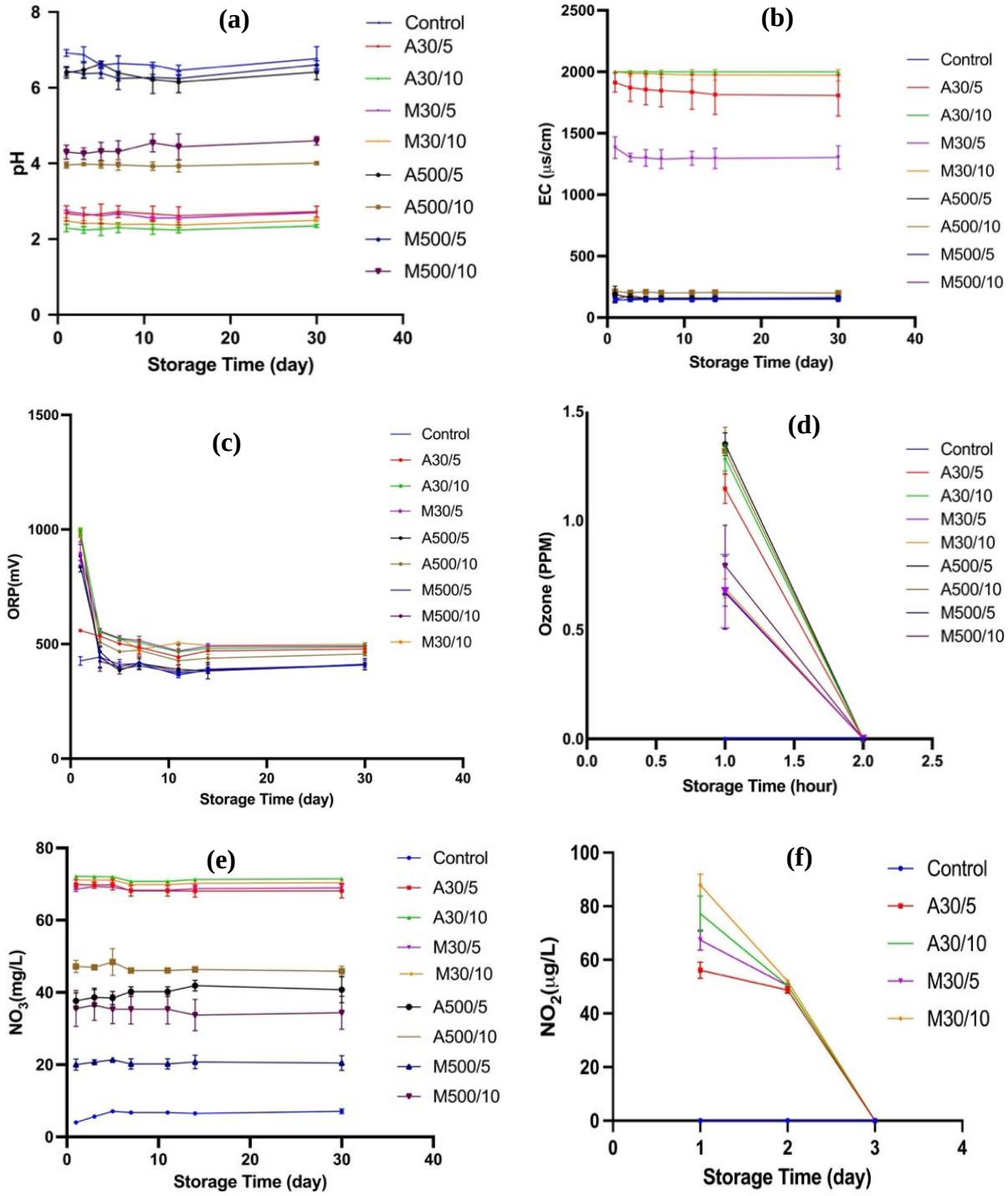

Figure. 3.2 Effect of storage time on the stability of the physicochemical properties of PAW generated from simulated air (SA) (80% nitrogen and 20 % oxygen) and modified air (MA) (90% nitrogen and 10% oxygen) as feed gases for various treatments at room temperature (22 ± 2 ºC). (a) change of pH; (b) change of EC; (c) change of ORP; (d) change of O_3; (e) change of NO_3^-; (f) Change of NO_2

CHAPTER 4

EFFECT OF PLASMA-ACTIVATED WATER ON PEA SEED SPROUTING AND QUALITY PARAMETERS OF SPROUTS[2]

[2] To be submitted to Food and Bioprocess Technology

Abstract

This study evaluated the effect of plasma-activated water (PAW) on pea seeds and analyzed their physicochemical properties by treating potable water (PW) with DBD plasma. A total of 30 mL of PW were treated with atmospheric pressure cold plasma (ACP) with two gas compositions: simulated air (SA; 80% N_2 + 20% O_2) and modified air (MA; 90% N_2 + 10% O_2) for various treatment times 1, 2, 3, 4, 5, and 10 min resulting atmospheric plasma-activated water (APAW) and modified atmospheric plasma-activated water (MAPAW). For seed treatment, seeds were immersed in the water and treated. The electric conductivity (EC), oxidation-reduction potential (ORP), nitrite (NO_2^-), nitrate (NO_3^-), and ozone (O_3) significantly increased with treatment time, while the pH decreased in both APAW and MAPAW treatments ($p < 0.05$). Notably, H_2O_2 in all samples were below the detection limit. After treatment, the nitrate content of PW showed a nearly 17-fold increase after 10 min of treatment for both gas compositions. Seed germination was positively affected by the PAW treatment. Seeds treated with APAW4 and MAPAW4 showed a 75% increase in germination percentage. However, no significant effect on total phenol, flavonoid content, and antioxidant capacity. The gamma-aminobutyric acid (GABA) content shd a slight increase after PAW treatment ($p > 0.05$). Scanning electron microscopy analysis revealed that PAW treatment removed the waxy layer from the surface of pea seeds, which may help in sprouting. These results indicate that PAW can significantly improve seed sprouting. However, the treatment conditions need to be optimized for different seed types.

Keywords: Plasma-activated water (PAW), Atmospheric cold plasma (ACP). Seed sprouting; physicochemical properties.

Introduction

Recently, the consumption of plant sprouts in daily diets has noticeably increased due to their high nutritional value (Aloo et al., 2021; Benincasa et al., 2019; Galieni et al., 2020; Gan et al., 2017). Various seed sprouts, such as mung bean, alfalfa, pea, broccoli, and radish, are widely used as food ingredients worldwide. Additionally, these sprouts are classified as functional foods because of their high nutrient and phytochemical content. These sprouts are commonly consumed raw or gently cooked, often added to salads and sandwiches, and used as garnishes in culinary presentations. During sprouting, various physiological processes occur, such as the breakdown and metabolism of proteins and carbohydrates and the production of small molecular nutrients like amino acids, vitamins, and phenolic compounds. These significant changes happen in the seed composition, ultimately resulting in the growth of sprouts at harvest (Guiyun et al., 2022; Ikram et al., 2021). The germination or sprouting process enhances nutrition and reduces the adverse effects of anti-nutritional elements (Liu et al., 2022; Mir et al., 2021). Generally, the sprouting process for most edible seeds takes between 3 and 5 days; this slow process creates an environment conducive to microbial growth (Vidal-Valverde et al., 2002). It is necessary to shorten the sprouting time, reduce the microbial load, and enhance seed germination and the germination index to enhance the process. In recent years, various physical, chemical, and combined methods, such as gamma irradiation, electrolyzed oxidizing water, ultrasound, cold plasma, and pesticides, have been used to stimulate seed germination and promote sprout production. However, it has been noted that some of these technologies may have negative effects. As consumers increasingly prefer minimally processed and safe food products that retain a fresh taste, the demand for safety and freshness has led to extensive research to discover innovative and safe processes to improve the quality and safety of sprouts.

In this study, we focused on pea seeds (*Pisum sativum* L.) to explore the effects of Plasma Activated Water (PAW) on their germination and growth. Pea seeds are nutrient-rich, containing proteins, carbohydrates, minerals, and vitamins, yet there has been limited research on the influence of PAW specifically on them (De Almeida Costa et al., 2006; Rathore et al., 2022). This investigation is particularly relevant as pea seeds contribute to sustainable agriculture by

minimizing dependence on synthetic nitrogen fertilizers through their symbiotic relationship with nitrogen-fixing bacteria, such as rhizobia (Abeysingha et al., 2024). By understanding how PAW affects pea seeds, this study aims to enhance their growth and nutritional value, ultimately promoting more sustainable agricultural practices.

As a result, the development of non-thermal processing systems has been a focus for many food researchers. Atmospheric cold plasma (ACP) has emerged as a promising non-thermal technology among these novel technologies. With the development of non-thermal plasmas at low and atmospheric pressures, plasma applications in agriculture and food processing are expanding rapidly. Many studies have directly applied ACP to seeds to improve their germination rates (Ling et al., 2014; Liu et al., 2022; Sadhu et al., 2017; Zhou et al., 2016). However, the irregular surface of food products presents various hidden areas unexposed to cold plasma treatment.

To overcome this problem, plasma-activated water (PAW) was used as an alternative method to stimulate seed germination, serving as an eco-friendly and economical option. PAW has recently exhibited remarkable biological activity in agronomy and medicinal applications (Guragain et al., 2021). Instead of direct seed treatment by ACP, the treatment of seeds with PAW is drawing attention due to PAW's effectiveness as an indirect and efficient application. PAW is a liquid created by exposing water to a plasma source in the water or above its surface (Thirumdas et al., 2018). This interaction leads to alterations in the physical and chemical characteristics of the water, including a drop in pH and a rise in oxidation-reduction potential (ORP) and electrical conductivity (EC) caused by the generation of primary and secondary reactive species. Various reactive oxygen and nitrogen species, including H_2O_2, O_3, NO_2^-, and NO_3^-, are generated based on the feed gas, discharge type, and plasma chemical composition. The presence of these reactive species in PAW gives it potential as a disinfectant for eliminating pathogens (bacteria, fungi, viruses, pests, etc.), selectively targeting cancer cells, preserving food, promoting seed germination and microbial inactivation, and more (Guo et al., 2021; Rathore et al., 2022; Thirumdas et al., 2018). Several studies have investigated the use of plasma for preserving fruits and vegetables, decontaminating meat and eggs, and treating various products (Benabderrahim et al., 2024; Ozen et al., 2024; Pal et al., 2016; Sharma & Singh, 2022; Wang et al., 2021). Recently, PAW has been widely used to enhance plant quality during seed germination and growth, with evidence showing its impact on seed germination and growth (Adhikari et al., 2020; Fan et al., 2020; Liu et al., 2019; Lo Porto et al., 2018; Mildaziene et al., 2022; Sivachandiran & Khacef, 2017; Zhang et al., 2017).

It has been observed that the long-lived reactive nitrogen species (RNS) and reactive oxygen species (ROS) dissolved in PAW significantly enhance plant growth (Billah et al., 2020). Nitrate in PAW can improve plant growth and may serve as an alternative to chemical fertilizers. However, the mechanisms behind these effects are still not fully understood.

This study aimed to examine PAW's impact on the sprouting of pea seeds with a focus on optimizing gas composition and treatment duration. Pea sprouts are known for their high nutritional value, including protein, carbohydrates, vitamins, and minerals, and are widely consumed worldwide. However, the influence of PAW on pea seed sprouting and nutritional quality has been less explored. Therefore, this research aims to achieve the following objectives:

1. Determine the physicochemical properties of PAW.
2. To assess the effect of PAW seed germination, sprouting time, and the phytochemicals and bioactive compounds in the sprouts.
3. Investigate PAW's role in altering pea seeds' surface morphology and wettability properties.

Materials and Methods

Pea seeds (*Pisum sativum L.*) were obtained from Mountain Valley Seed Co.(E-commerce) and were stored at room temperature (22°C ± 2 °C) until used. The seeds were handpicked to ensure uniform shape, size, and color and were close to circular shape. All the analytical grade chemicals were obtained from the Sigma-Aldrich Co. (St. Louis, MO).

Atmospheric pressure cold plasma treatment (ACP)

This study used an ACP device (CD50, plasmatreat) to generate plasma, as illustrated (Sharma & Singh, 2022). The DBD reactor comprises a power generator (FG5001) operating at 1 kV, with a frequency range of 15- 25 Hz and a current of 16 A, in conjunction with a high-voltage transformer (HTR11), as detailed (Ozen et al., 2022). The plasma jets use oxygen and nitrogen to produce plasma by applying an electric field across the electrodes. The plasma is ejected from a nozzle head, and the active species are blown out like a small jet. The DBD reactor consists of a tube-shaped glass apparatus that directs the gas through the nozzle outlet, creating an unstable cavity in water.

PAW preparation

The ACP device was utilized to generate PAW with two different gas mixtures: (i) 80% nitrogen and 20% oxygen, designated as simulated air (SA), and (ii) 90% nitrogen and 10%

oxygen, referred to as modified air (MA). During the experiment, 30 mL of potable water (PW) was added to a 50 mL beaker placed beneath the plasma nozzle. PAW was generated at atmospheric pressure and fed into the PW via a PTFE (polytetrafluoroethylene) tube of 120 mm connected to the nozzle outlet. This setup ensured direct interaction between the plasma species and the water, allowing uniform mixing of reactive species. The treatment durations varied as 0, 1, 2, 3, 4, 5, and 10 min, labeled as control, APAW-1, APAW-2, APAW-3, APAW-4, APAW-5, APAW-10 for the simulated air composition, and MAPAW-2, MAPAW-3, MAPAW-4, MAPAW-5, MAPAW-10 for the modified air composition. All experiments were carried out at a gas flow rate of 10 L/min. The resulting PAW was used to evaluate its effects on pea seed sprouting.

Measurements of physicochemical properties of PAW

The physicochemical properties of PAW were measured using a pH meter (Hanna Edge) for pH, a conductivity meter (Hanna HI-981303, Romania) for electric conductivity (EC), and an ORP meter (Hanna Instruments HI98120, Romania) for oxidation-reduction potential (ORP). The nitrate (NO_3^-), nitrite (NO_2^-), and hydrogen peroxide (H_2O_2) concentrations were determined spectrophotometrically using the methods described by Pandey et al. (2023).

For nitrate measurement, 3 mL of PAW was mixed with 60 µL of 1N HCl, and the final absorbance was calculated by subtracting twice the absorbance at 275 nm from the absorbance at 220 nm, using a nitrate standard solution (1 to 5 mg/L). Nitrite content was determined using the Griess reagent. In this method, 3 mL of PAW was combined with 120 µL of Griess reagent, incubated for 30 min, and the absorbance was measured at 543 nm. A nitrite standard solution (50 to 300 µg/L) was used for calibration.

H_2O_2 concentration was determined by mixing 1.5 mL of 0.1 M titanium oxysulfate with 1.5 mL of PAW, followed by a 30-minute incubation. Absorbance was then measured at 407 nm, with a 30% H_2O_2 solution (0.01 to 1 mM) used as the standard. O_3 concentration in PAW was measured using a dissolving ozone vacuum kit (K-7423: 0-500 ppm) (Ozen et al., 2022).

Pea Seed sprouting

A total of 30 pea seeds of similar size and shape were selected and placed inside the 30 mL of PW and treated with ACP at two gas compositions using SA and MA for varying treatments: 0, 1, 2, 3, 4, 5, and 10 min to generate APAW and MAPAW. After treatment, seeds were soaked in water in 30 mL for 10 h and incubated at room temperature (22 ± 2 °C) with relative humidity of

approximately 70% to promote sprouting. After soaking, the seeds were placed on 9-cm petri dishes layered with filter paper and incubated at (22 ± 2 °C). To maintain moisture during the germination, 10 mL of the respective PAW were added to their respective petric dish, and 5 mL of corresponding PAW was added thrice daily throughout the sprouting period. The graphical representation of PAW sprouting is shown in Figure 4.1.

The germination process was closely monitored, with the number of germinated seeds recorded daily. A seed was considered to have completely sprouted when the sprout emerged from the seed coat and reached 1.5 cm. Germination parameters were calculated using the following Equations (1-4) (Zhou et al., 2019).

After 3 days of germination, the pea seed sprouts were collected, and their length, fresh weight, and dry weight were measured. On the final day of germination, the total sprout length and the diameter of the hypocotyls were measured using vernier calipers.

$$Germination\ percentage\ (\%) = \frac{N_t}{N_{TS}}\ x\ 100 \quad (1)$$

$$Germination\ Potential(\%) = \frac{N_{3D}}{N_{TS}}\ x\ 100 \quad (2)$$

$$Germination\ Index\ GI = \frac{\sum ND_n}{D_n} \quad (3)$$

$$Vigor\ Index\ V_i = G_i\ x\ L \quad (4)$$

Where,

N_t: Number of seeds germinated after incubation time t.

N_{TS}: Total number of seeds in each petri dish.

N_{3D}: Number of germinated seeds per dish in the first three days.

N_{Dn}: Number of germinated seeds on the day n.

D_n: Germination Day.

L: length of the seedling.

Water uptake of seeds

Seed water uptake was assessed by measuring the seeds before and after soaking them in PAW, while untreated seeds were soaked in PW. Approximately 5g of seeds were weighed using an electronic scale (Mettler Toledo, Switzerland). The seeds were then immersed in 30 mL water and treated according to specified treatment times for each sample. The water uptake was measured in triplicate (n=3) to ensure consistency. The dry seeds were initially weighed as W_i. After plasma treatment, they were soaked in PAW for 2 h. Following soaking, the seeds were dried with a paper

towel to remove excess surface moisture, their final weight was recorded as W_f. The amount of water absorbed by the seeds was then calculated using Equation (5).

$$Wa\ (\%) = \frac{Wf - Wi}{Wi} x\ 100 \quad (5)$$

Preparation of sprout extract

Initially, sprouted peas were freeze-dried using a lyophilizer (Millrock Technology, REVO, USA) at -40 °C for 28 h. After freeze-drying, the sprouts were ground to a fine powder using a grinder. A 0.5 g of each sprout powder was extracted in a solvent mixture (acetone: water: acetic acid, 70:29.5:0.5, v/v/v) at a ratio of 1: 10 (w/v) at 22 ± 2 °C for 3 h, with shaking at 150 rpm (Junior orbit shaker, Lab-line, Illinois, USA). Following extraction, the mixture was centrifuged at 6800 X g for 10 min at 22 ± 2 °C. The resulting residue was re-extracted twice with 5 mL of the same solvent mixture. The supernatants were combined and stored at 4 °C for future analysis (Xiang et al., 2019).

Determination of total phenolic content

The total phenolic content of pea sprouts was determined using the Folin-Ciocalteu method, as described by (Xiang et al., 2019). Briefly, 0.1 mL of each sprout extract was mixed with 0.4 mL of distilled water and 0.1 mL of Folin-Ciocalteu's reagent solution and allowed to react for 6 min at room temperature (22 ± 2 °C). Following this, 1 mL of 7% (w/v) sodium carbonate solution and 0.8 mL of deionized water was added and incubated for 90 min at room temperature. The absorbance of each solution was measured at 760 nm using a spectrophotometer (Cole-Parmer Instrument Co., Chicago, USA). The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g dry weight).

Determination of total flavonoid Content

The total flavonoid content (TFC) was measured using an aluminium chloride colorimetric method (Tian et al., 2016). Briefly, 2 mL of the sprout extract was mixed with 0.4 mL of a (1:20) $NaNO_2$ solution and allowed to react for 6 min. Afterwards, 0.4 mL of a 1:10 $AlCl_3$ solution was added and incubated for 6 min. To the mixture, 4.5 mL of 1 mol/L NaOH was added, and the final volume was adjusted to 10 mL with distilled water. The absorbance was measured at 510 nm using

a spectrophotometer (Cole-Parmer Instrument Co., Chicago, USA). The results were expressed as milligrams of rutin equivalents (RE) per gram of dry weight of sprouts (mg RE/g dry weight).

DPPH scavenging activity

The DPPH**.** Scavenging activity in pea sprout extracts was measured using the method described (Xiang et al., 2019). Briefly, 300 µL of pea sprout extract was mixed with 3.9 mL of a 60 µM DPPH radical solution in ethanol. The reaction mixture was incubated for 30 min at room temperature (22 ± 2 ° C) in the dark. After incubation, the absorbance was measured at 517 nm using a spectrophotometer (Cole-Parmer Instrument Co., Chicago, USA). The DPPH scavenging activity was calculated using the following equation:

$$\text{DPPH. scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x100} \quad (6)$$

$A_{control}$ is the absorbance of the DPPH standard solution, and A_{sample} is the absorbance of the DPPH solution incubated with pea sprouts.

Determination of gamma-aminobutyric acid content

About 0.1 g of freeze-dried sprout powder was added to an Eppendorf tube containing 2 mL of 70% aqueous ethanol. The mixture was vortexed for 1 min, then ultrasonicated at 10 °C for 25 min and centrifuged at 34,600 x g for 20 min. This extraction process was performed twice, and the resulting supernatants were combined and stored at −20 °C for analysis.

For derivatization, a 1 mL aliquot of the above extract was mixed with 0.6 mL of borax buffer (pH 8) and 1 mL 2-hydroxynaphthaldehyde (0.3% w/v in methanol) (Khuhawar & Rajper, 2003). The mixture was heated in a water bath at 80 °C for 10 min, then cooled to room temperature. The final volume was adjusted to 5 mL with 50% methanol.

A calibration curve for GABA was constructed using 0, 25, 50, 75, and 100 µg/mL GABA. The derivatized GABA samples were analyzed using ultra-high-performance liquid chromatography (UHPLC, Acquity, Waters, Milford, Massachusetts, USA) with a photodiode array (PDA) detector. Chromatographic separation was achieved on a BEH C18 column (1.7 µm, 2.1 × 50 mm),

with a binary mobile phase consisting of 0.1% aqueous formic acid and 100% acetonitrile. The gradient elution program, as outlined by Wang et al. (2015), was applied over a 9 min run. The derivatized GABA peak was detected at 330 nm.

Chemical and morphological characterization of seeds

FTIR spectroscopy

The physicochemical properties of sprout powders before and after PAW treatment were assessed using the Thermo Scientific Nicolet 6700 FTIR spectrometer. The FTIR, equipped with an ATR (attenuated total reflectance) crystal, and spectra were acquired over the wavenumber range of 400–4000 cm^{-1}. A total of 64 scans were collected for each sample, with background subtraction applied prior to data acquisition.

Scanning Electron Microscopy Investigation

Seeds treated with PAW and untreated seeds were analyzed to examine their surface structure. Pea seeds were soaked in the designated water for 8 h at room temperature (22 ± 2 °C). After soaking, the seeds were removed, and any excess water was absorbed using a paper towel. They were then fully dried in a vacuum oven (Precision Scientific Inc., Illinois, USA) at 70 °C for 72 h. Morphological analysis was conducted using a Hitachi SU-3900 scanning electron microscope (SEM).

Statistical analysis

Experiments were conducted using three replicates. Data analysis was performed using GraphPad Prism version 10.2.2 (GraphPad Software, San Diego, CA, USA), employing analysis of variance (ANOVA) to evaluate the results. Statistical significance was established at ($p < 0.05$), with mean differences assessed using Tukey's multiple comparison test.

Results and Discussion

Evaluation of physicochemical properties of PAW as a function of feed gas and treatment time

Several studies have shown that the properties of PAW are influenced by various plasma parameters, including discharge type, pressure, electrode distance, power supply, type of working gas and flow rate, treatment duration, and sample volume (Kučerová et al., 2019; Thirumdas et al., 2018; Zhou et al., 2019). This study employed two feed gases (SA and MA) to generate PAW. An effective approach to optimizing concentrations of reactive species involves generating plasma in the gas phase and bubbling it through the liquid, similar to our experimental setup (Sivachandiran & Khacef, 2017). Plasma generates a variety of short-lived species, such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical (·OH), peroxynitrite ($ONOO^-$), and nitric oxide (NO·). Upon interaction with water, these species react to form more stable end-products like NO_2^-, NO_3^-, and H_2O_2, which are long-lived species of interest. To elucidate the plasma-induced acidification of PAW, we used spectrophotometric methods to measure the concentrations of NO_2^-, NO_3^-, O_3, and H_2O_2 in both PW and PAW. The results are illustrated in Table 4.1. All measurements were conducted immediately after PAW generation.

The results show that the concentrations of these reactive species in PAW increase with longer plasma treatment times. Among the ROS, only O_3 was detected, while H_2O_2 was not observed in any of the treatments. Initially, O_3 was absent in the PW, but after treatment, its concentration increased to 0.92 ± 0.06 and 0.83 ± 0.03 ppm after 1 min of treatment, reaching maximum levels of 1.28 ± 0.06 ppm and 0.69 ± 0.04 ppm for the 10-min treatments of APAW and MAPAW, respectively.

In addition to the ROS, RNS was also generated during PAW production. The data revealed a linear increase in nitrate and nitrite concentrations as treatment time extended. Specifically, nitrate levels rose from 4.02 ± 0.30 mg/L to 59.01 ± 7.30 mg/L in APAW and 51.85 ± 1.83 mg/L in MAPAW after 1 min, further increased to 71.70 ± 1.11 mg/L and 69.17 ± 1.20 mg/L after 10 min of plasma treatment, respectively. Nitrite concentrations increased from 0 to 73.33 ± 0.23 μg/L

in APAW and 85.73 ± 4.23 μg/L in MAPAW following 10-min treatments. These findings align with previous reports by Oehmigen et al. (2010).

Table 4.2 demonstrates a significant decrease in the pH of PAW, with a reduction from 6.92 ± 0.08 to 3.66 ± 0.24 for APAW and 5.01 ± 0.66 for MAPAW after just 1 min. After 10 min of treatment, the pH dropped to 2.29 ± 0.08 and 2.47 ± 0.09, respectively. Notably, no significant differences were observed between the APAW and MAPAW treatments. Additionally, a substantial increase in electrical conductivity (EC) was noted, with values rising from 147.33 ± 17.01 μS/cm to < 2000 μS/cm for APAW and 1719.33 ± 269.29 μS/cm for MAPAW after 10 min of treatment. The oxidation-reduction potential (ORP) also increased from an initial value of 439.667 ± 41.18 mV to 527.33 ± 23.69 mV for APAW and 526.00 ± 2.00 mV for MAPAW. These results indicate the accumulation of active ions due to plasma treatment, and higher concentration was found in APAW samples compared to MAPAW.

In our current study, the H_2O_2 is below the detection limit in the PAW despite its known stability as a long-lived species. The lack of detectable H_2O_2 in our study may be attributed to its rapid decomposition by nitrates under acidic conditions (Lukes et al., 2014; Machala et al., 2013). Previous research indicated that H_2O_2 levels could drop significantly shortly after treatment due to low pH and other factors, with reductions from 2000 mg/L to 10 mg/L occurring within 10 min of water treatment with DBD treatment (Shainsky et al., 2012). In this study, the electrodes were not immersed in water to generate PAW, and longer exposure times may have influenced H_2O_2 availability in our samples. This contrasts with findings from the N_2/O_2 gas mixture study (citation here), which reported that an 80% N_2 and 20% O_2 mixture yielded the highest H_2O_2 concentrations, while a 50% N_2 and 50% O_2 mixture had the highest nitrate levels. Previous studies also demonstrated that varying H_2O_2 levels depend on parameters such as treatment volume and electrode-liquid gap; for instance, a study by Vaka et al. (2019) reported up to 50 mg/L of H_2O_2 after just 3 min of exposure with a 20 mm gap. The small volumes and small gaps used in other studies led to significantly different H_2O_2 concentrations, highlighting the importance of these parameters in PAW generation and chemical composition (Ikawa et al., 2010; Traylor et al., 2011). Notably, leading PAW generation systems for H_2O_2 production effectively utilize water droplets, which help sequester H_2O_2 in the liquid phase and minimize decomposition reactions triggered by

radicals from the gas and at the interface. These findings emphasize the critical roles of pH, exposure time, and gas composition in maintaining H_2O_2 stability and other reactive species concentration during PAW generation.

Effect of PAW on seed sprouting

During sprouting, seeds initially absorb water, which activates the biochemical process that triggers the seed enzyme system and converts stored carbohydrates into soluble sugars and fatty acids for the development of the embryo (Chen et al., 2023). Following the embryo cells' expansion, the radicle protrudes through the seed coat. To evaluate the effects of APAW and MAPAW on seed sprouting, pea seeds were treated with PAWs for three days, with PW used as the control. The germination rates, determined by radicle emergence, showed that PAW treatments enhanced seed germination and seedling growth compared to PW. Figure 4.2 shows the final day images of sprouts. Specifically, germination percentages increased to 70% and 69.5% for APAW and MAPAW, respectively, while PW only reached about 40% ($p < 0.05$). This represents a 75% improvement over the control, which is shown in Figure 4.3. In addition to this, the germination potential also improved, with approximately 77% of seeds germinating in PAW treatments compared to around 65% with PW. Other germination parameters are shown in Table 4.3. On the last day of germination, the average lengths and diameters of the sprouts were measured, revealing no significant differences among treatments. Figures 4.4 (a) and 4.4 (b) present the average measurements as approximately 12 mm in length and 7 mm in diameter. These findings suggest that while plasma treatment, particularly the RONS like nitrates and nitrites, may enhance germination rates, they do not significantly impact length and diameter. Similarly, no significant difference is observed in other germination parameters, which may be because of less penetration of reactive species of PAW into the seeds.

The increase in germination is attributed to NO_2, NO_3, and H_2O_2 species in PAW. While H_2O_2 is known to act as a signalling molecule during germination, its positive effects were not evident in our study, which aligns with (Wang et al., 2023). Instead, NO_3^- and NO_2^- likely served as nitrogen sources for the pea seeds, influencing germination by being metabolized or reduced to nitric oxide (NO), which modulates hormones such as abscisic acid and gibberellins RONS are crucial signalling molecules that regulate various developmental processes in plants. The required

concentrations of these species can significantly impact growth and development. Recent reviews emphasize the role of NO in seed germination and plant growth, linking it to the positive effects of abscisic acid (ABA) in regulating seed dormancy (Arc et al., 2013). NO_3^- serves as a major nitrogen source, and its reduction to NO_2^- by nitrate reductase (NR) produces nitrogenous metabolites beneficial for growth. In particular, NO_3^- promotes the release from dormancy and subsequent germination in many plant species, suggesting that NO_3^- generated by DBD plasma in PAW significantly enhances germination rates.

However, a limited range of beneficial reactive nitrogen species (RNS) concentrations varies depending on the seed type; excessive and insufficient amounts can impede crop growth. For instance, Fan et al. (2020) reported that nitrate (NO_3^-) concentrations exceeding 20 mg/mL did not promote growth in mung beans, with a decline in germination rate observed at 118.39 mg/mL levels. On the other hand, Lo Porto et al. (2018) reported that PAW containing 1.24 or 10.54 mg/L of NO_3^- positively influenced soybean seed germination. In our study, the total concentrations of NO_2^- and NO_3^- were below 80 μg/L and 80 mg/L, respectively, with all PAW treatments enhancing pea seed germination to varying degrees, except for the 10-min treatments of APAW and MAPAW. Notably, PAW-4 exhibited a significant increase in germination percentage. Our findings also revealed a good correlation between germination rate and RNS concentrations, suggesting that germination velocity directly depended on the RNS in PAWs.

Water uptake of seeds

Figure 4.5 displays the water uptake of seeds exposed to various treatments and controls. Although treating the seeds with PAW improved the germination rate compared to PW, it did not affect the seeds' water uptake. The seeds demonstrated increased water absorption in the initial 2 h of immersion, but the results were not significantly different ($p>0.05$). Similar results were found by Rathore et al. (2022). This lack of significant effect may be due to the hard structure of the seed coat or the concentration of reactive species in the PAW, which may not have been sufficient to crack the seed within the initial two hours. Several studies suggest that the acidic nature of PAW can accelerate the erosion of the seed coat, allowing for higher water uptake as the seed coat cracks (Billah et al., 2020; Chen et al., 2023; Fan et al., 2020; Than et al., 2022). Thus, the increased water

absorption in PAW-treated seeds is believed to enhance germination by facilitating nutrient absorption from the water.

Effect of PAW on total phenolic and flavonoid contents of sprouts

As reported in Table 4.4, pea sprouts' total phenolic content (TPC) and total flavonoid content (TFC) were measured after the end of sprouting. There was no significant difference in the TPC and TFC of sprouts treated with PAW and PW ($p > 0.05$). Previous findings by Guo et al. (2017); Xiang et al. (2019) showed similar results. Typically, the increase in the phenolic and flavonoid compounds in seeds is a defensive response against oxidative stress induced by the PAW. However, Mildaziene et al. (2018) found an increase in phenolics of cone flower seeds after short exposure, attributing this to a defense mechanism against unfavorable conditions. In our study, however, the RONS may not have induced a sufficient level of oxidative stress to give a noticeable increase in the secondary metabolites.

Antioxidant assay

Table 4.4 shows the changes in antioxidant values. The DPPH scavenging assay is a widely used spectrophotometric method for assessing antioxidant capacities. No significant differences in antioxidant potential were observed between PAW-treated seeds and the control (PW) ($p > 0.05$). Comparable results were observed (Xiang et al., 2019; Xu et al., 2016). Typically, the ROS in PAW can activate the antioxidant enzymes, which help protect cells from oxidative damage. Under typical conditions, PAW exposure would trigger these enzymatic pathways, leading to an increase in antioxidant activity (Mandici et al., 2022). However, in the present study, no such increase was observed, likely due to the relatively low concentration of ROS in the PAW used.

Effect of PAW on Gamma-aminobutyric acid (GABA)

GABA is a non-protein amino acid synthesized via the decarboxylation of L-glutamic acid by the enzyme glutamic acid decarboxylase (GAD; EC 4.1.1.15). Research indicates that GABA metabolism plays a crucial role in helping plants manage external stress. Higher GABA levels support metabolic processes, such as plant defense against environmental stresses such as acidosis, mechanical damage, extreme temperatures, drought, and insect herbivory. In stressful conditions,

GABA accumulates in plant tissue, regulating cellular reactions, metabolic activities, and gene transcription, enabling quick adaptation to stress (Chou et al., 2021).

The increase in GABA content is not linear with treatment time, as shown in Figure 4.6; it increased insignificantly by 0.54 times compared to the control. The seeds treated with APAW 2 showed the highest GABA content. The results were aligned with the research conducted (Chou et al., 2021), and it was found that GABA content was highest in the 80W treatment, which is 2.98 times greater than the untreated.

The increase in GABA levels does not follow a linear pattern to the relative power applied. Another study by Wang et al. (2023) observed that PAW treatments enhanced GABA levels of wheat seeds compared to control samples, attributing this effect to osmotic and oxidative stresses induced by low pH and the presence of nitrite (NO_2^-) and nitrate (NO_3^-). Variations in GABA levels among different PAW treatments were likely due to differential stress responses. These factors create a unique stress environment that may vary in intensity, leading to differing levels of GABA accumulation across treatments. Furthermore, the reactive species present in PAW exert external pressure on the plant, stimulating GABA synthesis within the seeds, which results in increased levels of GABA after treatment with PAW.

On the other hand, a study by Wang et al. (2022) buckwheat sprouts found that the highest GABA content was observed on the 9th day of germination compared to seeds, nearly three times that of the seeds. However, no significant difference was found between plasma-treated and control groups. While the underlying mechanism remains unclear, further research is needed to elucidate how plasma treatment affects GABA production.

Physicochemical properties of pea sprouts

To analyze the physicochemical properties of sprout powder, we used FTIR spectroscopy to examine its chemical bonds. We compared the results before and after treatment with PAW, as shown in Figures 4.7 (a) and 4.7 (b), illustrating the typical ATR spectra of pea sprout powder.

The identical FTIR spectral patterns suggest that the chemical compounds in the pea sprouts are quite similar. The O-H bond represents water, polysaccharides, and proteins in the

spectrum and ranges from 3500-3000 cm^{-1} (Ďurčányová et al., 2023). The spectrum exhibited characteristic bands indicative of lipids, with symmetric and asymmetric stretching of C–H bonds in the 2980–2820 cm^{-1} range. The C=O stretching vibration varies based on the bond's location and structure, with peaks from unionized carboxylate carbonyl stretching appearing at 1730 cm^{-1}, while amide I and cellulose features can be found at 1650–1640 cm^{-1}. The presence of proteins is suggested by amide I at approximately 1640 cm^{-1} and amide II at around 1540 cm^{-1}. In the 1200–900 cm^{-1} range, a strong band with overlapping peaks represents polysaccharide bonds, the highest at 995 cm^{-1} and the lowest peak at 860 cm^{-1} resembles hemicellulose and lignin (Karunakaran et al., 2020; Švubová et al., 2020).

The spectra of all treatments showed close similarities; only peaks displayed different intensities, which indicates that no new groups were formed and no chemical changes occurred after treatment with PAW. Comparing the plasma treatment and control groups, we observed an increase in the hydroxyl (OH) stretching vibrations of polysaccharides and C-H bond stretching of lipids. This enhancement is likely due to the reactive oxygen and nitrogen species (RONS) generated during plasma treatment, which induces oxidative modifications of the lipids and polysaccharides on the surface of the sample. The mild nature of non-direct plasma is likely to minimize the harmful effects and damages to the sprouts compared to direct cold plasma. The results show that PAW may be a safer alternative for sprouting compared to direct plasma treatment without significantly changing the seeds' physical and chemical properties.

Surface morphology of pea seeds after PAW treatment

Figures 4.8 (a) and 4.8 (b) show that treating pea seeds with PAW significantly alters their surface structure. Untreated pea seeds have a consistent pattern of bumpy protrusions. After PAW treatment, these protrusions become increasingly eroded, resulting in a notably rougher surface. This treatment effectively removes the hydrophobic waxy layer on the seeds, which normally delays water absorption.

Figures 4.8 (a) and 4.8 (b) depict the natural wax covering the seed surface, making it hydrophobic. In contrast, PAW-treated seeds exhibit improved hydrophilicity. The presence of RONS, including H_2O_2, dissolved O_3, and NO_3^-/NO_2^- ions, plays a critical role in oxidizing and

eliminating this waxy layer. Similarly, previous studies reported that PAW treatment helped remove wax from the seeds' surface, eroding the bumpy protrusions with plasma treatments (Billah et al., 2020; Rathore et al., 2022).

The mechanism involved in this process is that plasma treatment targets the outer hydrophobic waxy layer, known as the cuticle, which consists of long-chain saturated hydrocarbons and oxygen-containing groups. This plasma treatment erodes the waxy material and makes it hydrophilic. For example, air DBD plasma significantly eroded the rough waxy surface of black gram seeds (Billah et al., 2020). In other legume species, the hardness of the seed depends on the seed coat and cuticle present; the loss of cuticle helps in seed softening. Moreover, the physical dormancy of pea seeds is linked to the impermeable barrier between the waxy cuticle and the underlying macrosclereid cells, known as the "light line."

Additionally, the treatment of mung bean seeds in water with atmospheric pressure PAW showed eroded and cracked seed surfaces (Zhou et al., 2019). This research underscores the potential of PAW treatment to enhance seed properties by modifying surface characteristics. However, interestingly, no significant increase in water uptake is observed. Based on the findings, the plasma may affect the outer seed surfaces, enhancing imbibition and reducing hardness.

Conclusion

The pea seeds were placed inside the PW and subjected to simulated and modified air treatment. The results demonstrated a strong correlation between the RONS in the PAW, gas composition, and treatment time. The PAW was effective in increasing the germination percentage of pea seeds and showed no significant effects on the antioxidant capacities, phenol content, flavonoid content, and GABA. Seeds treated with APAW4 and MAPAW4 exhibited a higher germination percentage than the control group. The process parameters, including gas composition, flow rate, discharge power, liquid volume, and seed type, significantly influence the outcomes of cold plasma treatment. Future investigations to determine the optimal processing conditions are essential, as these may vary across plant species. The results of this study serve as a useful reference for using PAW treatment to improve the quality of sprouts and germination rate.

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Table 4.1 Physicochemical properties (NO_3, NO_2, and O_3) of PAW of various treatments. Different lowercase letters indicate significant differences ($p < 0.05$). ND: Not detected; APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Treatment time (min)	NO_3 (mg/L)		NO_2 (µg/L)		O_3 (PPM)	
	APAW	**MAPAW**	**APAW**	**MAPAW**	**APAW**	**MAPAW**
0	4.02 ± 0.304^{f}		ND		ND	
1	59.92 ± 7.30^{d}	51.85 ± 1.83^{e}	50.26 ± 0.46^{f}	$52.13 \pm 1.15^{e, f}$	$0.92 \pm 0.06^{c, d}$	$0.83 \pm 0.03^{d, e}$
2	$66.12 \pm 0.18^{a, c}$	$60.75 \pm 0.78^{c, d}$	50.40 ± 0.00^{f}	$51.60 \pm 0.69^{e, f}$	$1.03 \pm 0.03^{b, d}$	0.58 ± 0.09^{f}
3	$68.02 \pm 0.31^{a, b}$	$65.36 \pm 1.24^{b, c}$	$51.20 \pm 0.00^{e, f}$	$56.66 \pm 0.23^{d, f}$	$1.03 \pm 0.02^{b, d}$	$0.67 \pm 0.07^{e, f}$
4	$69.14 \pm 0.59^{a, b}$	$66.98 \pm 0.07^{a, c}$	$52.53 \pm 1.61^{e, f}$	$65.73 \pm 0.46^{b, c}$	$1.06 \pm 0.05^{b, c}$	0.59 ± 0.06^{f}
5	$69.93 \pm 0.59^{a, b}$	$67.96 \pm 0.07^{a, b}$	$60.00 \pm 0.69^{c, d, e}$	$64.80 \pm 2.77^{b, d}$	$1.14 \pm 0.06^{a, b}$	$0.67 \pm 0.16^{e, f}$
10	71.80 ± 1.11^{a}	$69.17 \pm 1.20^{a, b}$	73.33 ± 0.23^{a}	85.73 ± 4.33^{b}	1.28 ± 0.06^{a}	$0.69 \pm 0.04^{e, f}$

Different lowercase letters indicate significant differences ($p< 0.05$). ND: Not detected; APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Table 4.2 Physicochemical properties (pH, EC, ORP) of PAW of various treatments. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Treatment time (min)	pH		EC(μS/cm)		ORP (mV)	
	APAW	MAPAW	APAW	MAPAW	APAW	MAPAW
0	6.92 ± 0.08 [a]		147.33 ± 17.01 [g]		439.66 ± 41.18 [b]	
1	3.66 ±0.24 [c]	5.01 ± 0.66 [b]	282.66 ± 58.70 [f, g]	199.66 ± 18.14 [g]	496.66 ± 49.23 [a, b]	494.00 ± 41.18 [a, b]
2	3.03 ± 0.08 [d, e]	3.26 ± 0.04 [c, d]	525.66 ± 23.11 [e, f]	318.66 ± 19.14 [f, g]	507.66 ± 38.68 [a, b]	505.00 ± 3.60 [a, b]
3	2.76 ± 0.06 [d, f]	3.02 ± 0.16 [d, e]	793.33 ± 23.09 [c, e]	522.33 ± 70.60 [e, f]	514.00 ± 34.64 [a, b]	513.00 ± 3.60 [a, b]
4	2.67 ± 0.02 [d, f]	2.82 ± 0.03 [d, f]	1033.00 ± 139.28 [c]	695.33 ± 35.44 [d, e]	518.66 ± 30.92 [a, b]	517.66 ± 2.51 [a, b]
5	2.57 ± 0.07 [e, f]	2.70 ± 0.12 [d, f]	1352.66 ± 156.18 [b]	926.33 ± 42.19 [c, d]	522.33 ± 26.31 [a, b]	522.00 ± 3.00 [a, b]
10	2.29 ± 0.08 [f]	2.47 ± 0.09 [e, f]	< 2000 [a]	1719.33 ± 269.29 [a]	527.33 ± 23.69 [a]	526.00 ± 2.00 [a]

Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water; EC = electric conductivity; ORP= Oxidation reduction potential.

Table 4.3 Germination characteristics of pea sprouts of various treatments. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Treatment time (min)	Germination Potential (%)		Germination Index (%)		Vigour Index	
	APAW	MAPAW	APAW	MAPAW	APAW	MAPAW
0	64.44 ± 9.62 [a]		41.48 ± 6.94 [a, c]		424.45 ± 101.34 [a, c]	
1	68.88 ± 15.03 [a]	71.1 ± 9.62 [a]	52.40 ± 11.44 [a, c]	55.37 ± 4.48 [a, b]	677.71 ± 187.22 [a, c]	603.59 ± 162.14 [a, c]
2	77.78 ± 1.92 [a]	73.33 ± 10.00 [a]	60.92 ± 3.39 [a]	58.88 ± 8.23 [a, b]	858.40 ± 101.57 [a, b]	890.20 ± 351.84 [a, b]
3	73.33 ± 10.00 [a]	79.99 ± 5.77 [a]	58.33 ± 6.93 [a, b]	61.11 ± 5.00 [a]	747.91 ± 200.30 [a, c]	955.36 ± 230.02 [a]
4	76.66 ± 12.01 [a]	76.66 ± 5.77 [a]	60.55 ± 10.01 [a]	59.44 ± 5.35 [a, b]	848.44 ± 242.32 [a, b]	901.06 ± 206.38 [a]
5	76.66 ± 6.66 [a]	73.33 ± 3.33 [a]	59.44 ± 7.28 [a, b]	50.55 ± 8.01 [a, c]	651.39 ± 81.40 [a, c]	520.60 ± 247.56 [a, c]
10	58.89 ± 10.18 [a]	58.89 ± 10.71 [a]	31.85 ± 7.08 [c]	38.52 ± 2.62 [b, c]	252.64 ± 105.89 [c]	325.98 ± 113.15 [b, c]

Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Table 4.4 Phytochemical properties of pea sprouts of various treatments. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

Treatment time (min)	Total Phenol Content (mg GAE/g DW)		Total Flavonoid Content (mg RE/g DW)		Antioxidant capacity (%)	
	APAW	MAPAW	APAW	MAPAW	APAW	MAPAW
0	$4.91 \pm 0.13^{a, c}$		$4.20 \pm 0.43^{a, b}$		88.94 ± 1.41^{a}	
1	$5.23 \pm 0.40^{a, c}$	5.63 ± 0.18^{a}	$3.91 \pm 0.25^{a, b}$	4.62 ± 0.08^{a}	90.04 ± 0.59^{a}	91.82 ± 0.19^{a}
2	$5.30 \pm 0.16^{a, c}$	$5.42 \pm 0.07^{a, c}$	$4.09 \pm 0.11^{a, b}$	$4.12 \pm 0.05^{a, b}$	89.94 ± 0.50^{a}	91.10 ± 0.94^{a}
3	$5.26 \pm 0.19^{a, c}$	$5.42 \pm 0.29^{a, b}$	$4.08 \pm 0.07^{a, b}$	$4.45 \pm 0.11^{a, b}$	89.22 ± 1.00^{a}	90.93 ± 1.01^{a}
4	5.56 ± 0.17^{a}	4.65 ± 0.20^{c}	$4.00 \pm 0.23^{a, b}$	$4.16 \pm 0.17^{a, b}$	88.83 ± 1.99^{a}	90.43 ± 1.20^{a}
5	5.55 ± 0.02^{a}	$4.74 \pm 0.50^{b, c}$	$3.98 \pm 0.28^{a, b}$	$3.98 \pm 0.21^{a, b}$	91.71 ± 0.44^{a}	91.10 ± 1.66^{a}
10	5.64 ± 0.45^{a}	$4.66 \pm 0.06^{b, c}$	3.91 ± 0.04^{b}	4.35 ± 0.29^{b}	91.81 ± 0.75^{a}	91.71 ± 0.00^{a}

Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water;

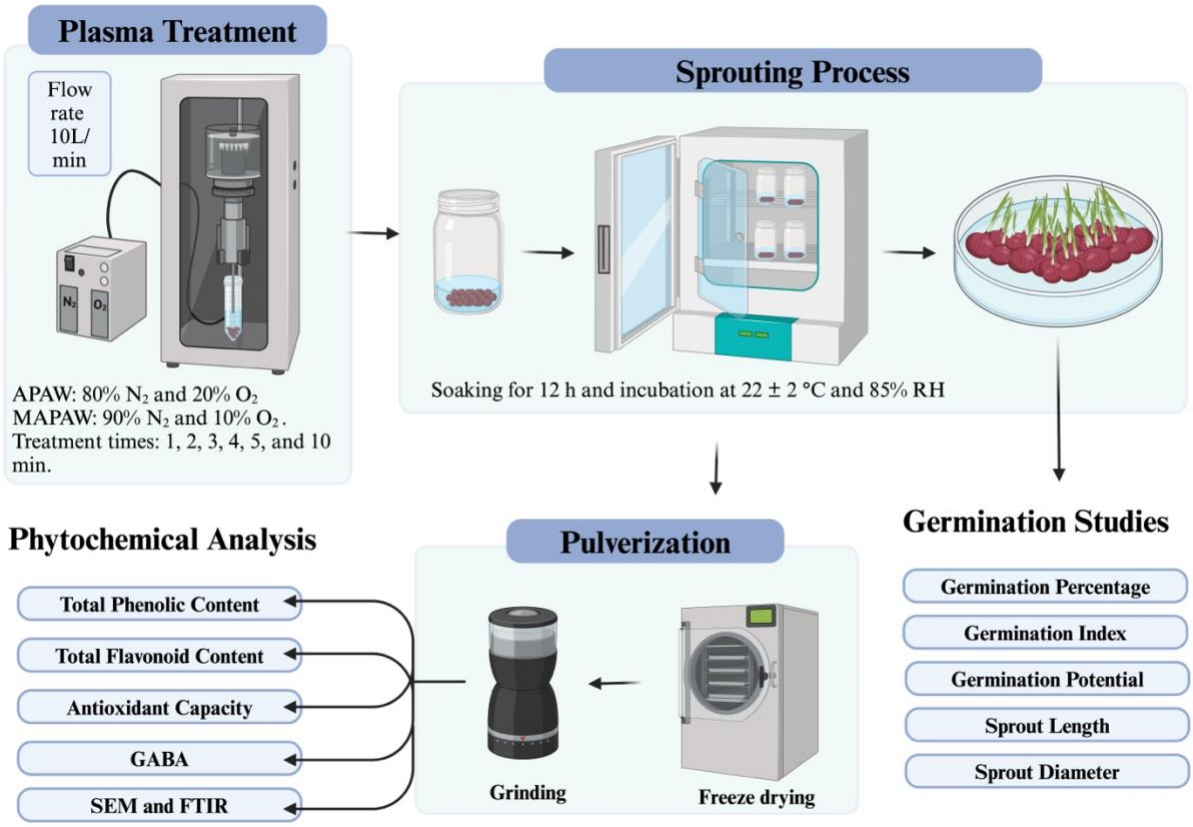


Figure. 4.1 Graphical representation of pea seed sprouting using plasma activated water.

Figure. 4.2 The effect of PAW on pea sprouting generated from simulated air (SA) (80% nitrogen and 20 % oxygen) and modified air (MA) (90% nitrogen and 10% oxygen) as feed gases for 4 min treatment. (a) Control; (b) APAW 4; (c) MAPAW 4.

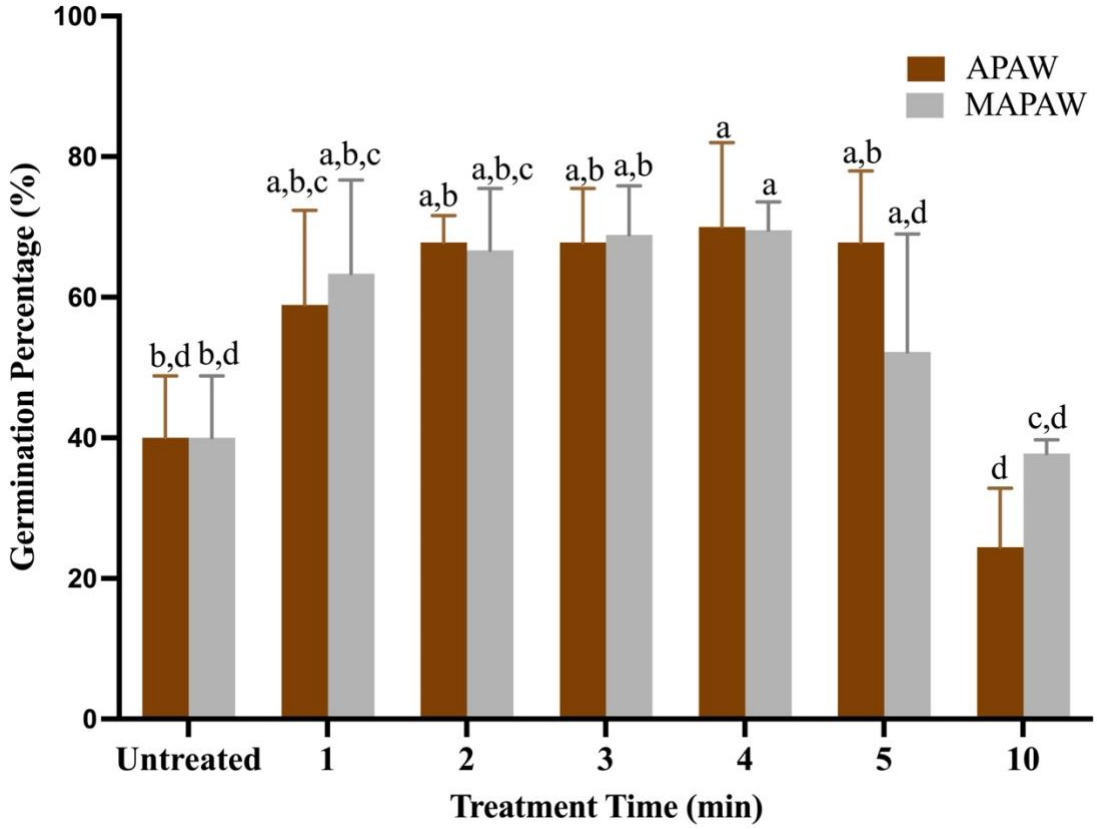


Figure. 4.3 The effect of APAW and MAPAW for various treatment times on Germination percentage. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

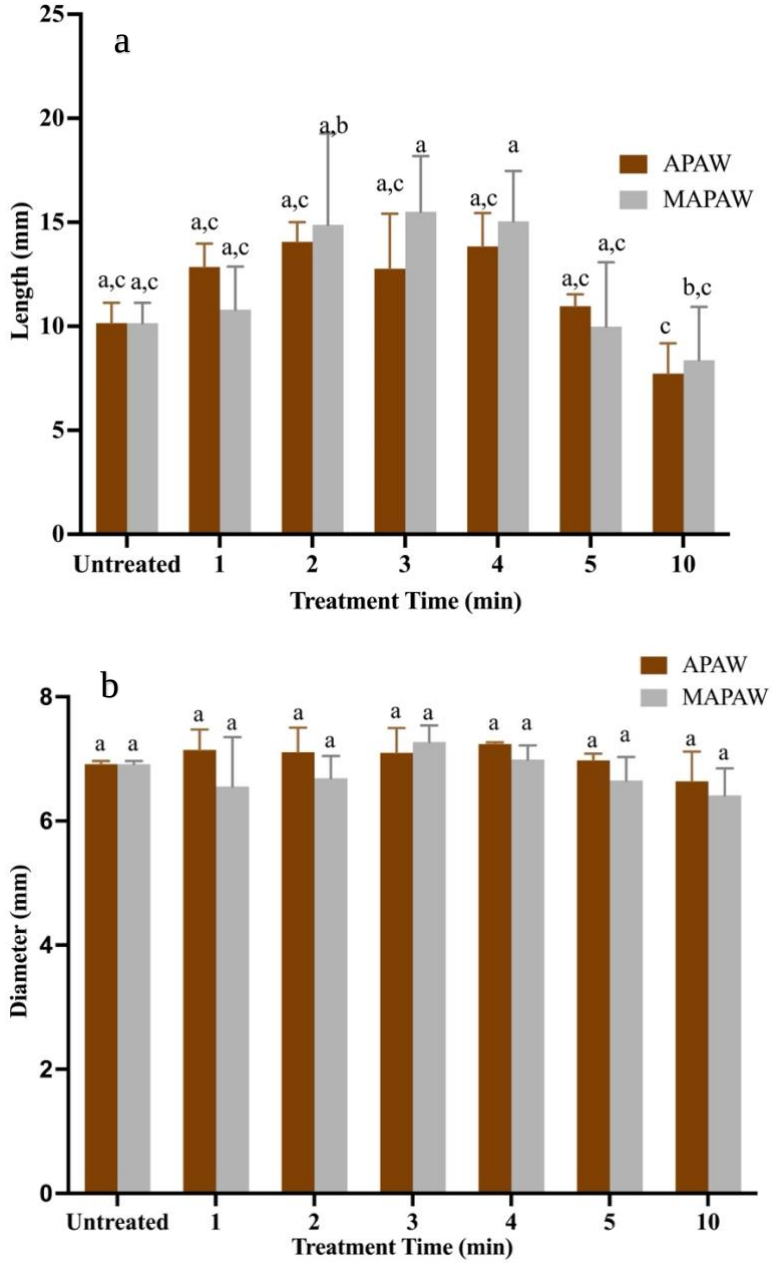


Figure. 4.4 The effect of APAW and MAPAW for various treatments on (a) Length and (b) Diameter. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water .

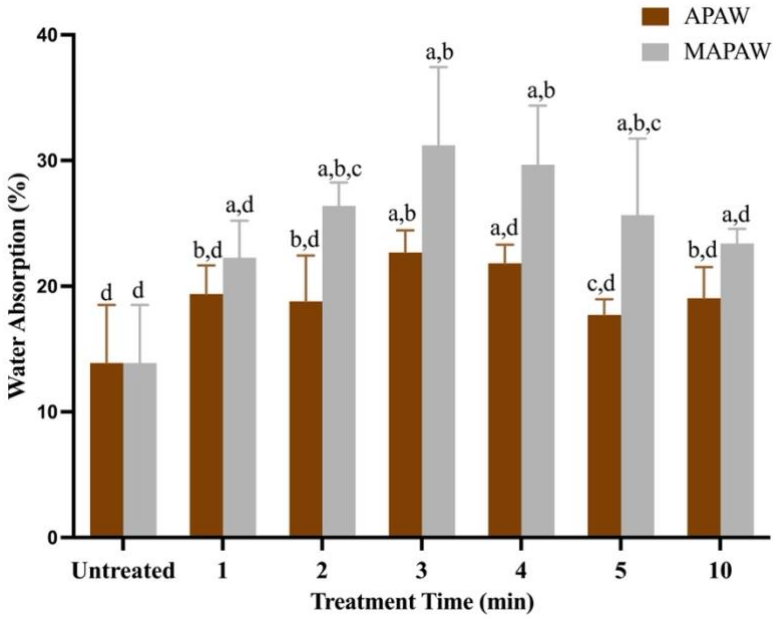


Figure. 4.5 The effect of APAW and MAPAW for various treatments on the water uptake of seeds. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water; MAPAW = Modified atmospheric plasma-activated water.

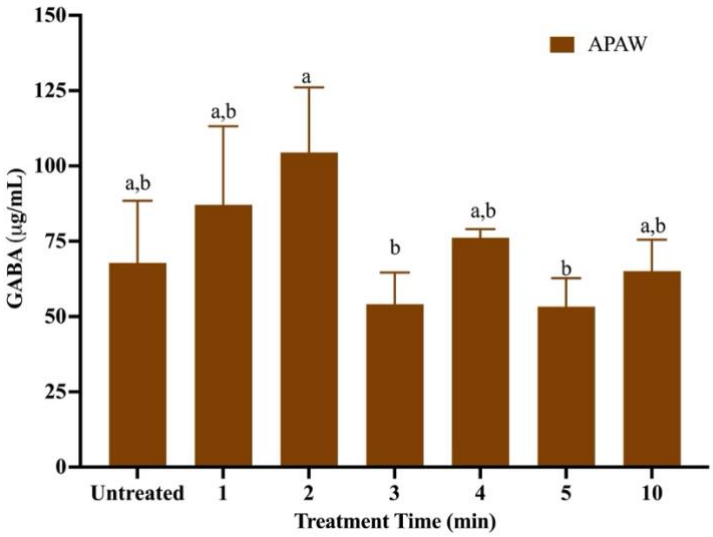


Figure. 4.6 The effect of APAW on the GABA. Different lowercase letters indicate significant differences ($p < 0.05$). APAW = Atmospheric plasma-activated water.

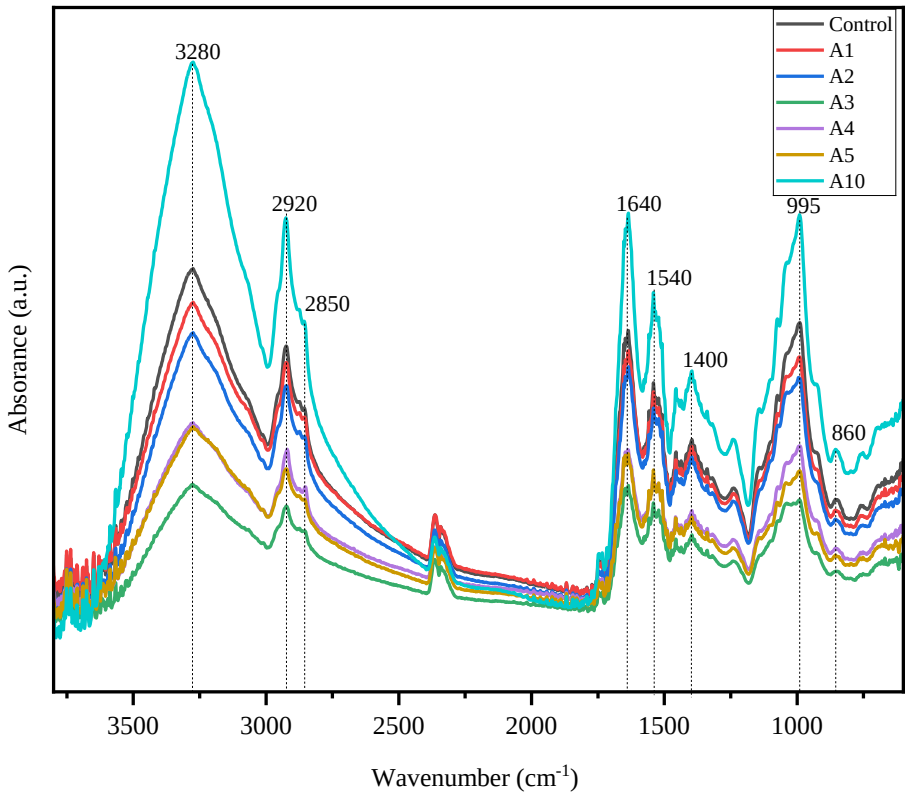


Figure. 4.7 (a)): FTIR spectrum of pea sprout powder treated with APAW. APAW = Atmospheric plasma-activated water

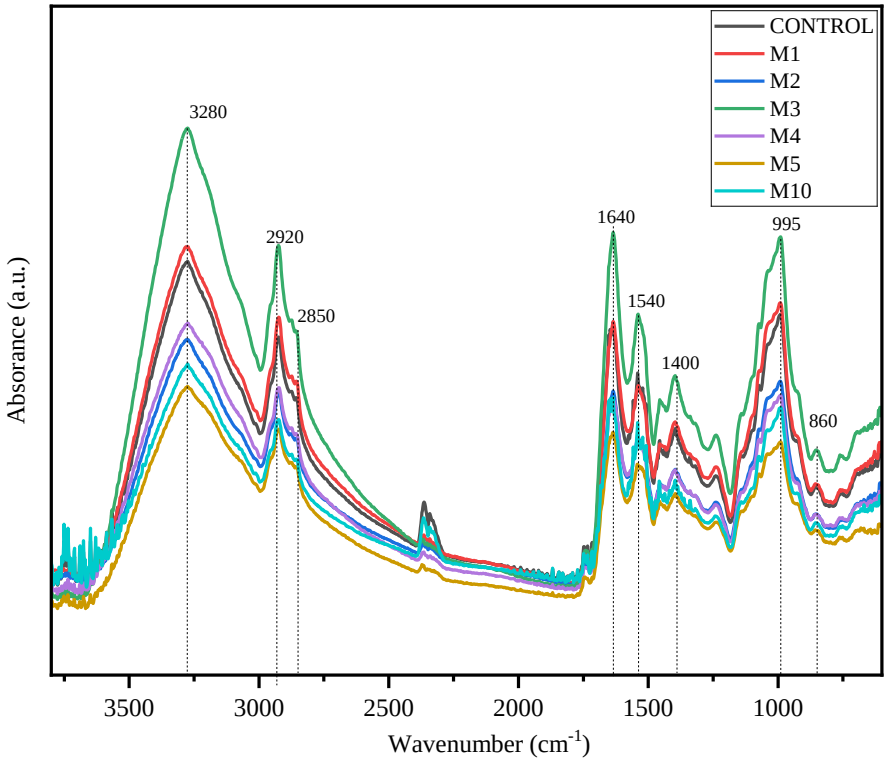


Figure. 4.7 (b) FTIR spectrum of pea sprout treated with MAPAW. MAPAW = Modified atmospheric plasma-activated wate

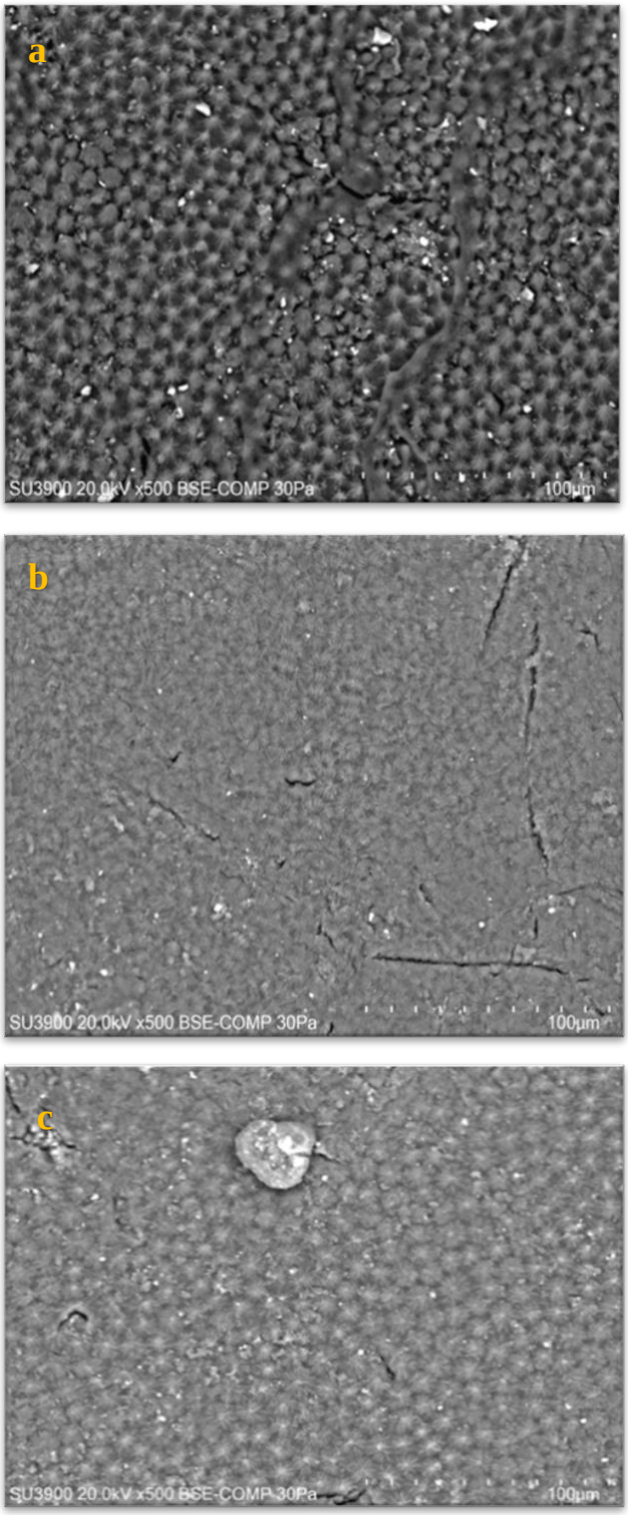


Figure. 4.8 SEM (100 µm) images of PAW-treated seeds using simulated air (SA) (80% nitrogen and 20 % oxygen) and modified air (MA) (90% nitrogen and 10% oxygen) as feed gases for 4 min treatment (a) Control and (b) APAW 4 (c) MAPAW 4 treatment.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

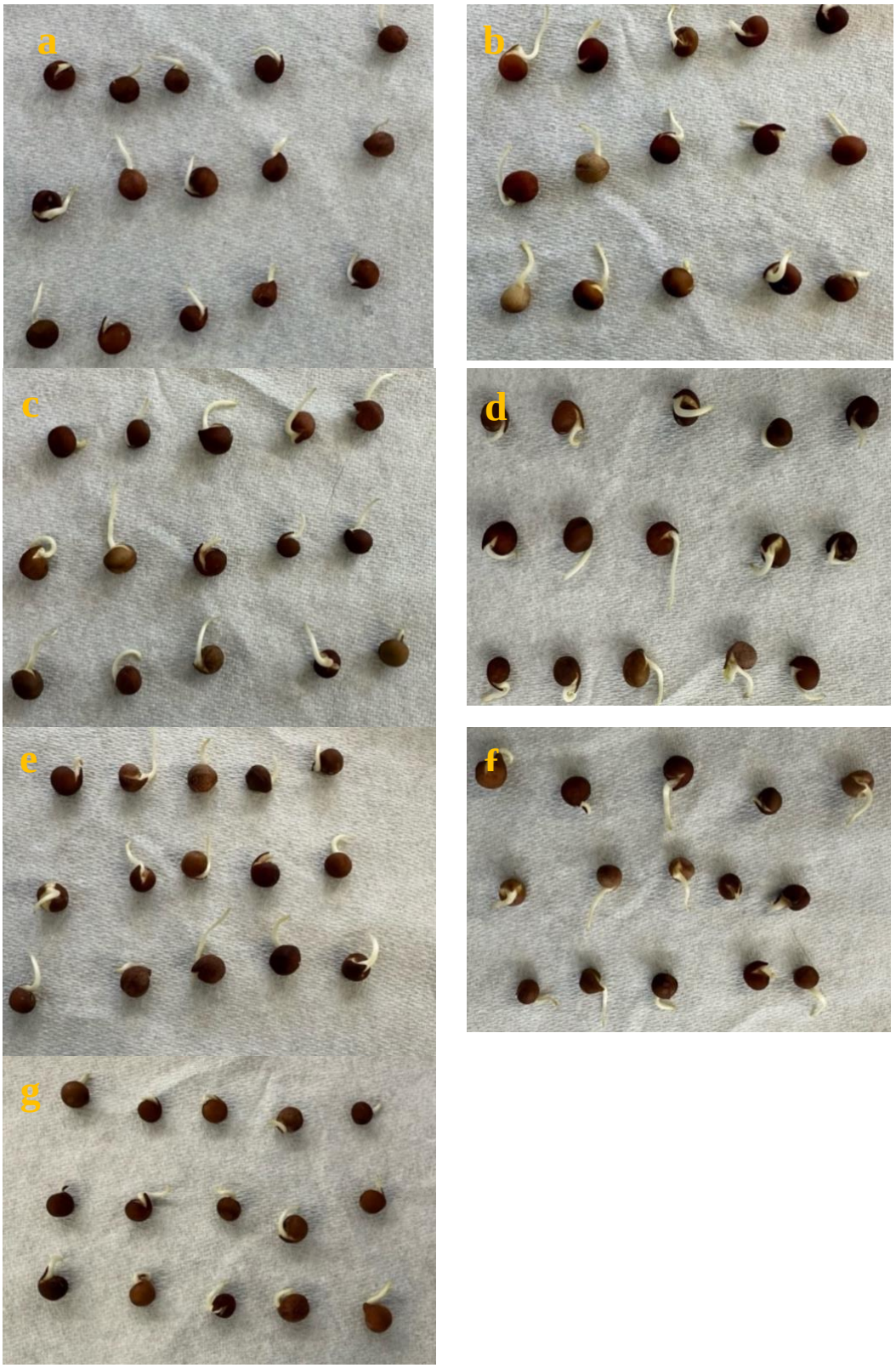
This study investigated the effects of plasma-activated water (PAW) on pea sprouting and the assessment of their quality. In Chapter 2, we examined how plasma treatment time, gas composition, and sample volume influence the physicochemical properties of PAW and its storage stability. Chapter 3 focused on the impact of these variables on pea seed sprouting and quality. Results indicated that the physicochemical properties of PAW remain stable over time, with an increase in reactive oxygen and nitrogen species (RONS) concentrations as treatment time and sample volume decrease. PAW treatment improved the germination percentage of pea seeds without adversely affecting their bioactive components.

Despite its promise as a non-thermal technology for seed germination and plant growth, several areas for future research are recommended to advance commercialization:

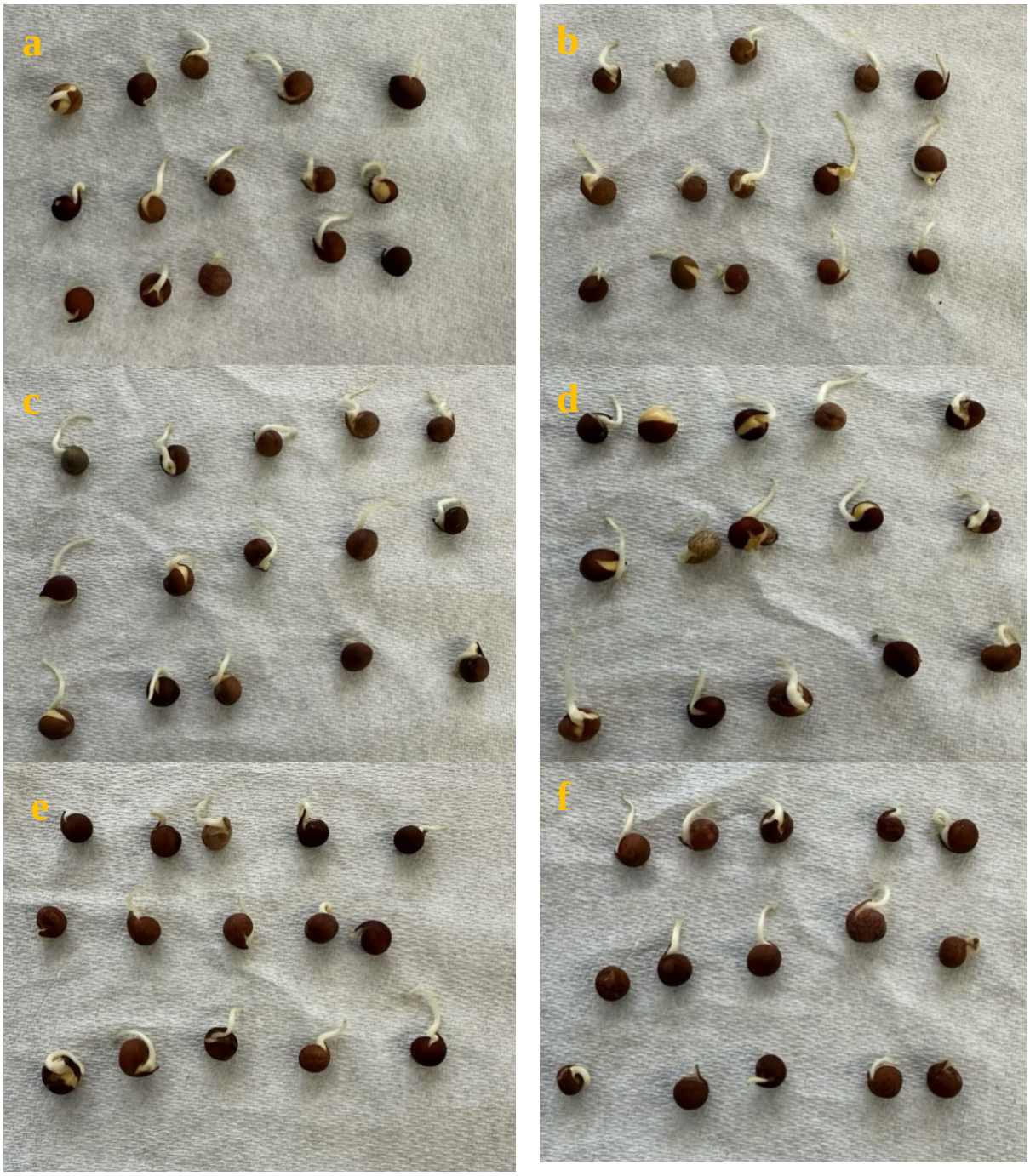
1. Comprehensive documentation of specific parameters used in cold plasma treatment—such as plasma source type, discharge power, treatment duration, sample volume, gas composition, and treatment distance, is essential. This will facilitate reproducibility and enable comparisons across studies.
2. While PAW has shown stability at room temperature (22 ± 2 ^{o}C), not all reactive species remain stable. Further research is needed to explore methods for preserving RONS at various storage temperatures. In our current study, H_2O_2 is not detected, and the nitrite level is low; further research is needed to
3. It is necessary to investigate the molecular effects of PAW on seed germination, as responses can vary by seed type. Optimizing RONS concentrations to enhance germination without negative impacts is crucial, as is assessing the long-term effects of PAW on food safety and sprout quality. Given that PAW has limited penetration into the interior of foods, its application should be complemented by other novel non-thermal technologies like ultrasound treatment and others. Future research could also explore the use of PAW in hydroponics to promote plant growth, expanding its potential applications in agriculture.

4. Consumer acceptance is vital for the success of food processing technologies. Sensory evaluations should be conducted to assess the impact of PAW on sprouts, focusing on taste, color, texture, and overall consumer perception to determine market acceptability.
5. Future research should evaluate the scalability and cost-effectiveness of implementing PAW technology in the food industry. This includes assessing the feasibility of integrating PAW systems into existing processing lines, estimating operational costs, and conducting cost-benefit analyses.

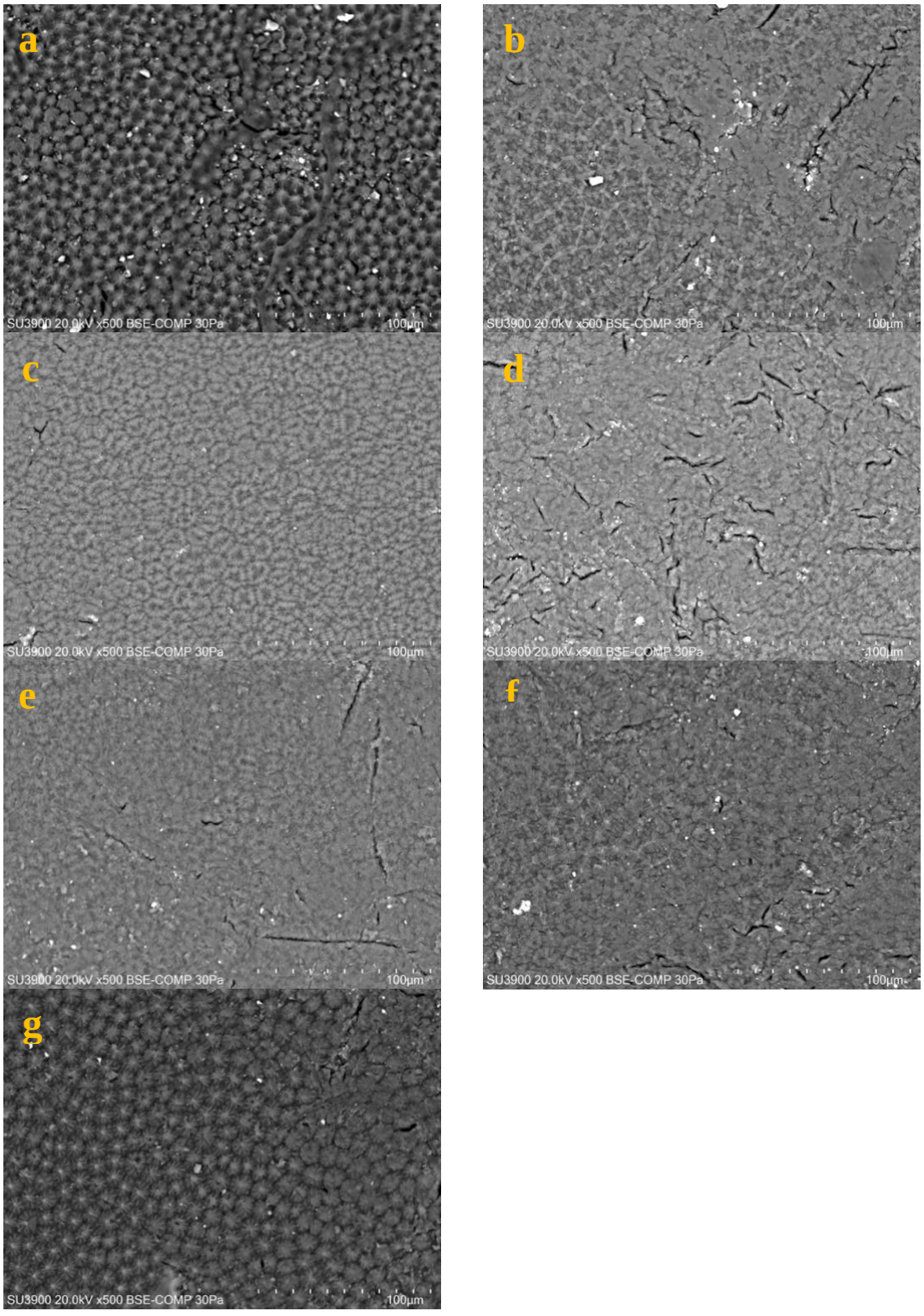
Addressing these future research directions will enhance PAW applications in sprouting, ultimately improving germination rates and the nutritional quality of sprouts.



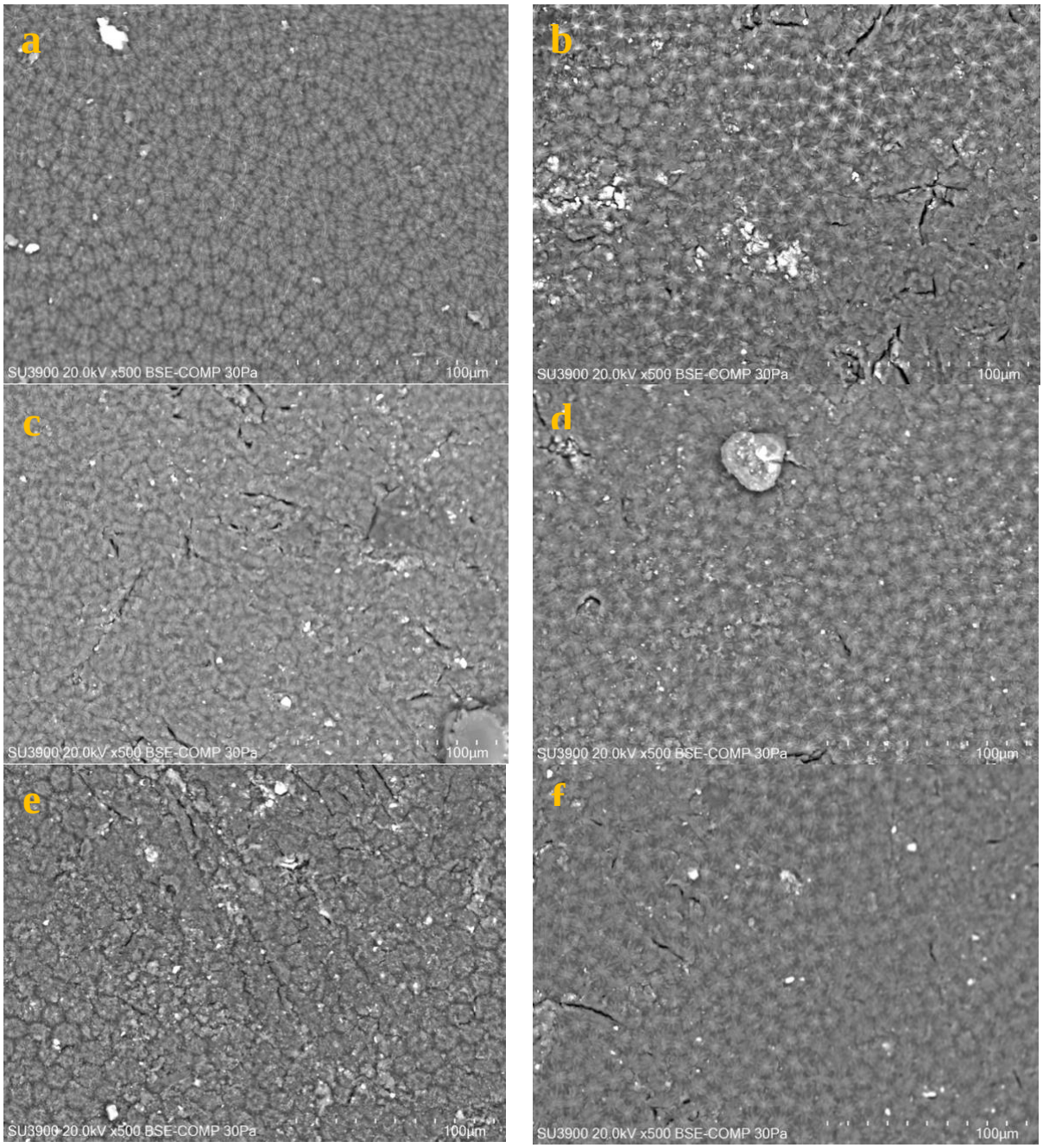
Appendix A. The effect of APAW on sprouting generated from simulated air (SA) (80% nitrogen and 20 % oxygen) as feed gases for various treatments: a) control, b) APAW 1, c) APAW 2, d) APAW 3, e) APAW 4, f) APAW 5, g) APAW 10. APAW = Atmospheric plasma-activated water.



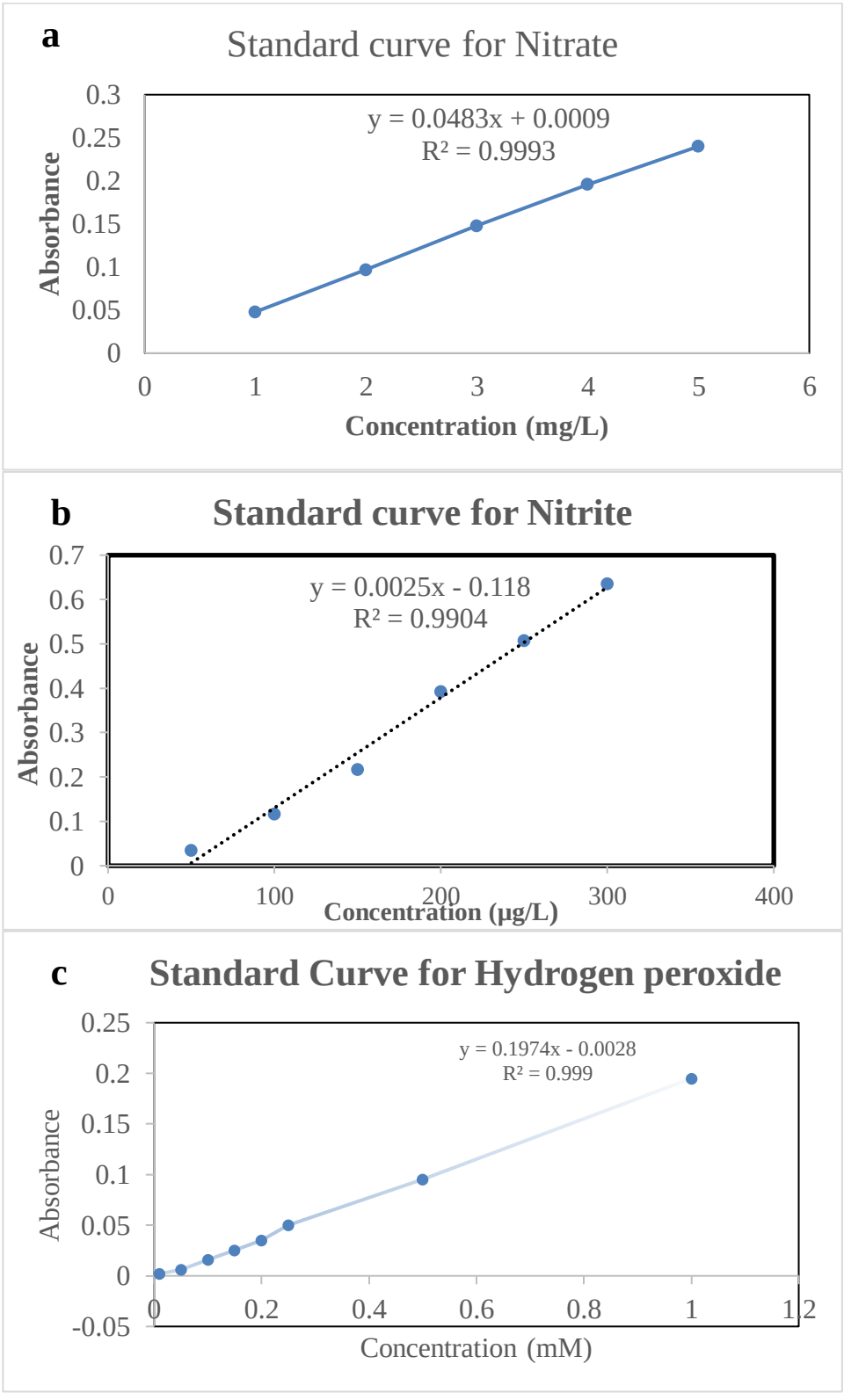
Appendix B. The effect of MAPAW on sprouting generated from simulated air (MA) (90% nitrogen and 20 % oxygen) as feed gases for various treatments: a) MAPAW 1, b) MAPAW 2, c) MAPAW 3, d) MAPAW 4, e) MAPAW 5, f) MAPAW 10. MAPAW = Modified atmospheric plasma-activated water

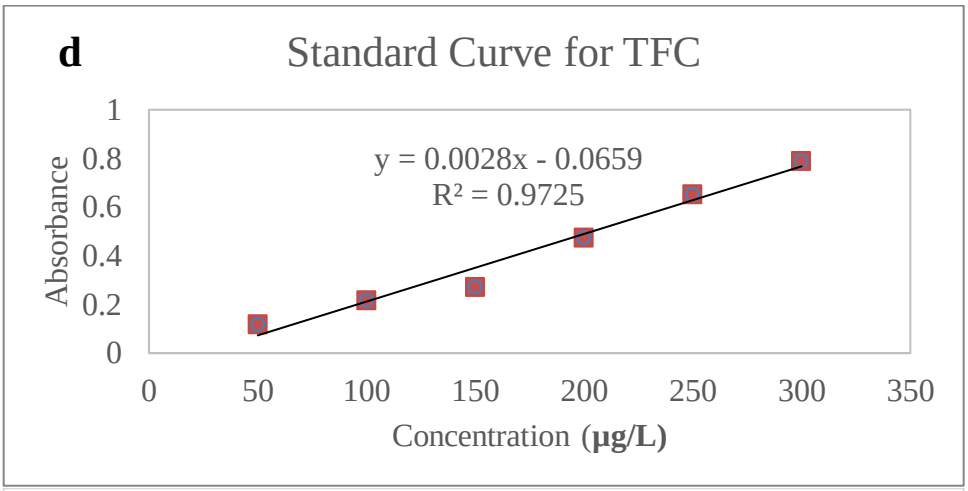


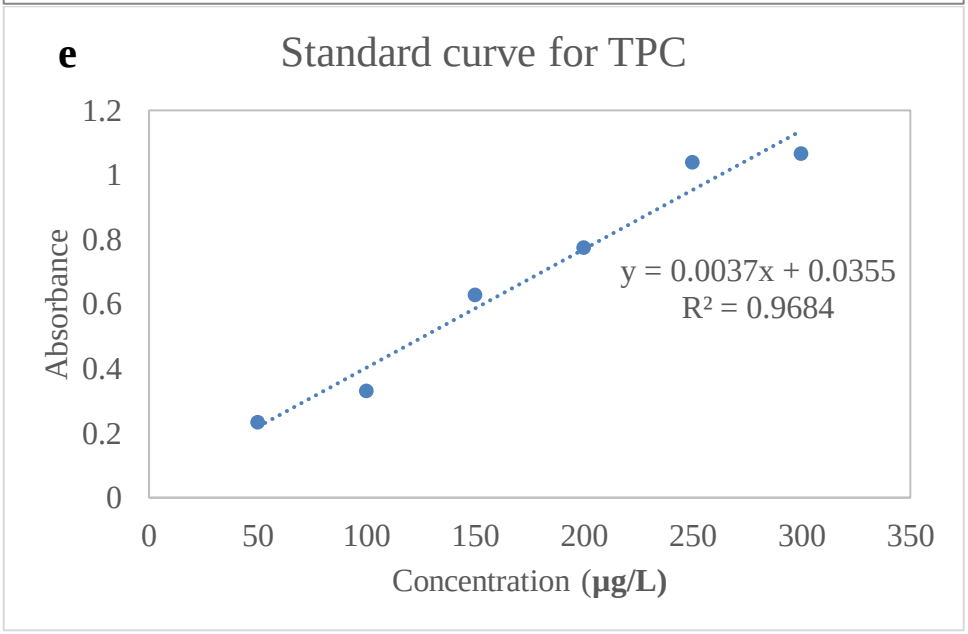
Appendix C. SEM Images (100 µm) of APAW treated seeds using simulated air (SA) (80% nitrogen and 20 % oxygen) as feed gases for various treatments: a) control, b)APAW 1, c) APAW 2, d) APAW 3, e) APAW 4, f) APAW 5, g) APAW 10. APAW = Atmospheric plasma-activated water.



Appendix D. SEM Images (100 µm) of MAPAW-treated seeds using modified air (MA) (90% nitrogen and 10 % oxygen) as feed gases for various treatments: a) MAPAW 1, b) MAPAW 2, c) MAPAW 3, d) MAPAW 4, e) MAPAW 5, f) MAPAW 10. MAPAW = Modified atmospheric plasma-activated water


a
Standard curve for Nitrate
y = 0.0483x + 0.0009
R² = 0.9993
Absorbance
Concentration (mg/L)
b
Standard curve for Nitrite
y = 0.0025x - 0.118
R² = 0.9904
Absorbance
Concentration (µg/L)
c
Standard Curve for Hydrogen peroxide
y = 0.1974x - 0.0028
R² = 0.999
Absorbance
Concentration (mM)





Appendix E. Standard curves: a) Nitrate, b) Nitrite, c) Hydrogen peroxide, d) Total flavonoid content e) Total phenol content.