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Enhancing germination and seedling growth of barley using plasma-activated water (PAW) with neutralized pH

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ABSTRACT

Achieving the highest seed germination and seedling growth rates is of paramount importance to maximize overall crop productivity. Among different technologies aiming at increasing seed germination and early plant growth, cold atmospheric pressure plasma (CAP) and plasma-activated water (PAW) are two of the most promising. However, CAP has been shown to decrease the pH of water, potentially constraining the utility of PAW in applications involving pH-sensitive plants. Here, we assessed the impact of magnesium addition to PAW (Mg-PAW), a potential mitigator of water acidity, on barley germination and growth compared to CAP technology without Mg (PAW). Although seed germination increased with both treatments compared to just DI water (control), the increase was higher when Mg-PAW was added, increasing by 2.29 and 2.59 times on day 2 and day 3. Application of Mg-PAW also increased water absorption, seedling growth (both in terms of weight and length), concentrations of chlorophyll, carotenoids, total soluble protein and enzymatic activities compared to both the control and the PAW treatment. The Mg-PAW displayed a 1.8-fold higher total soluble protein level compared to PAW alone. Although both treatments reduced Malondialdehyde (MDA) content, a prominent stress marker in plants, Mg-PAW application resulted in a 46% higher reduction in MDA content than PAW alone. Also, Mg-PAW application increased superoxide dismutase (SOD) activity by 50%, and catalase (CAT) enzyme activity by 8% compared to PAW alone. The implications of these discoveries extend to different agricultural applications, offering a promising avenue for improved early plant growth using Mg-PAW technologies under neutral or near-to-neutral pH conditions.

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1. Introduction

Agriculture is an interdisciplinary field that includes both the production of plants and the husbandry of animals for human consumption (Hu et al., 2022; Yi et al., 2022). Cold atmospheric pressure plasma (CAP) gained attention for its efficacy in biological applications (Samukawa et al., 2012; Han, Mumtaz, Ashokkumar, et al., 2022; Han, Mumtaz, & Choi, 2022; Han, Rana, et al., 2022; Mumtaz et al., 2022; Mumtaz, Khan, et al., 2023; Mumtaz, Rana, et al., 2023; Kim et al., 2024). The utilization of CAP to treat a variety of seeds has emerged as the key focus of research (Attri et al., 2021; Mildaziene et al., 2022; Jo et al., 2014; Ďurčányová et al., 2023). The efficacy of CAP technology to promote seed germination rate and plant growth increases the hope of this technology for an emerging field, Plasma Agriculture (Ranieri et al., 2021; Guragain, Baniya, et al., 2021). Understanding the knowledge of the field 'plasma medicine', the plasma agriculture field emerged (Qiu et al., 2023) and CAP technology development (Mumtaz et al., 2022; Babington et al., 2015). With its ability to transform atmospheric nitrogen into a variety of reactive nitrogen species (RNS), such as ammonia (NH_3) (Peng et al., 2018), nitric oxide (NO), nitrite (NO_2), nitrate (NO_3), di-nitrogen trioxide (N_2O_3) and dinitrogen pentoxide (N_2O_5), which can all be used as fertilizer (Van Alphen et al., 2021).

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Investigations on the CAP applications in the field of agriculture offer a detailed elucidation of its effectiveness on the plant life cycle (Yang et al., 2024), from seed germination to crop production (Ranieri et al., 2021; Guragain, Baniya, et al., 2021; Šerá et al., 2021). Over the past few decades, CAP technology has emerged as a potential technology that has the potential to produce various reactive species playing a key role as bio-signaling molecules in biological applications (Mumtaz et al., 2022). The induced reactive species play a vital role in the regulation of cellular metabolism, both in human and plant cells (Zhao et al., 2023). Exposing the normal water to CAP discharge leads to the formation of reactive species-enriched plasma-activated water (PAW). The irrigation of plants with PAW can possibility positively affect the plants to enhance their growth and productivity (Ito et al., 2018; Sivachandiran & Khacef, 2017; Šimečková et al., 2020; Sohan et al., 2021). The efficacy of PAW for such application is rooted in its unique characteristics, such as the availability of reactive nitrogen and oxygen species (ROS/RNS). The species in PAW has the potential to produce beneficial effects on plants. Generally, the availability of ROS/RNS in PAW can cause both beneficial or detrimental effects depending on the concentration (Medrano-Macías et al., 2022). At ideal or optimized concentrations, the reactive species of PAW act as bio-signaling molecules to influence various plant physiological processes for improved growth. On the other hand, high concentrations of ROS/RNS in PAW can become toxic and cause harmful or undesired effects after treatment (Khan et al., 2023). This phenomenon specifies the importance of the optimization of these ROS/RNS concentrations in every application scenario. The ROS/RNS have the potential to regulate directly and indirectly the gene expression levels in seeds, seedlings and plants (Gomes & Garcia, 2013; Grene, 2002). These species play a role in increasing the expression of genes linked to plant growth which eventually stimulates various growth-enhancing pathways, enzymatic activities and potential hormonal shifts to provide an environment for better plant growth (Priatama et al., 2022; Sajib et al., 2020; Zambon et al., 2020). It is already known that ROS/RNS of PAW, including hydrogen peroxide (H_2O_2) and NO_x, can positively impact plant growth (Kostoláni et al., 2021). The existence of H_2O_2 species with PAW irrigation can promote respiration, react with germination inhibitor markers and allow higher seed water absorption and faster germination rates. However, the RNS particularly NO_x species of PAW play a role as a nitrogen fertilizer to elevate the seedling growth (Adhikari et al., 2019; Stoleru et al., 2020). The PAW irrigation leads to the biosynthesis of various plant hormones associated with plant growth.

PAW generally can be produced with a low pH value due to H^+ ions in water (Judée et al., 2018). By soaking seeds or plant irrigation directly with this low pH PAW may have an undesirable impact on crop yields, mainly among species of plants that are sensitive to low pH (Hajiboland et al., 2023; Lamichhane et al., 2021). Thus, the development of an alternative procedure that can generate nitrogen-rich water at neutral pH levels is required. To neutralize the acidic nature of PAW typically involves the addition of a suitable base to maintain the pH value. However, this required the addition of such bases which do not show any major influence on the ROS/RNS concentration in PAW and unwanted effects. Previously, a study reported the use of various metal ions to produce PAW with neutralized pH (Javed et al., 2023; Lamichhane et al., 2021). Essential metals, including magnesium (Mg), play a pivotal role in plant physiology. The integration of this process can be adapted to both soil-based and hydroponic cultivation systems. The incorporation of Mg into PAW (Mg-PAW) serves to neutralize pH, thereby potentially manifesting additional benefits of CAP in the realm of agriculture. This adaptation proves advantageous, particularly in applications involving pH-sensitive plants within agricultural contexts. To the best of our knowledge, no investigation was conducted to assess the impact of PAW and Mg-PAW (with neutralized pH) on barley seedlings, along with the associated underlying mechanisms.

2. Materials and methods

In this study, we have used the PAW (low pH) and Mg-PAW (neutralized pH) to observe their effect on the germination and growth of barley seedlings. A plasma jet with air as a feeding gas is used to prepare the PAW and Mg-PAW. The germination rate and seedlings' growth were observed. In comparison to the control group, both PAW and Mg-PAW demonstrated enhanced germination rates and seedling growth. Notably, Mg-PAW with neutralized pH exhibited a superior increase in these parameters compared to PAW alone. Figure 1 shows the scheme and methodology of the study.

2.1. Preparation and properties of PAW

In this investigation, a plasma jet was employed to generate plasma and for the preparation of PAW. The power electrode, measuring 5 cm in length, was

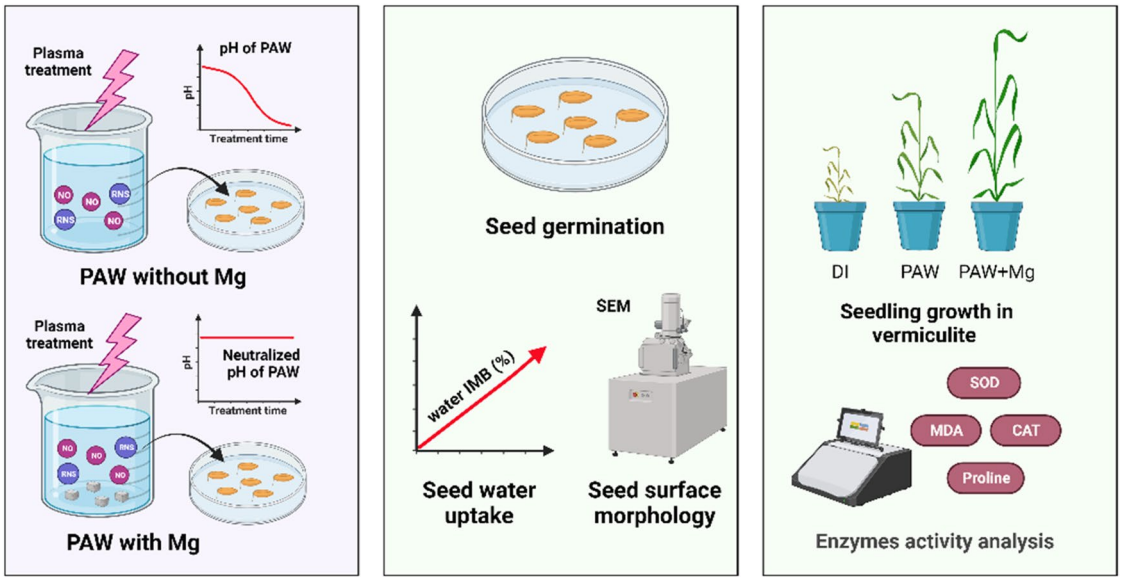

Figure 1. Schematic representation of the experimental design, methodology and analytical framework employed in the study.

positioned within a quartz tube with a length of 7 cm. A copper tape was attached to quartz which served as the ground electrode. An alternating current (AC) source, delivering a voltage of 10 kV, was applied to the power electrode while maintaining a gas flow rate of 1000 sccm to facilitate plasma generation. For the experimental setup, 50 mL of DI water underwent direct plasma treatment to produce PAW. The distance between the water's surface and the quartz tube's nozzle was meticulously maintained at a 5 mm distance. Additionally, a 4 g piece of Mg with 2 cm length and 1 mm thickness was immersed in the DI water during treatment to prepare Mg-PAW. The Mg is distributed in the water as sediment and stirred continuously during the preparation of PAW. Excessive use of Mg might lead to unwanted effects. In this work, we have optimized the correct amount of Mg which only played a role in neutralizing the pH (similar to DI water). Figure 2 provides a visual representation of the plasma source and preparation of PAW and Mg-PAW. Discharge voltage and current waveform measurements were conducted using a high-voltage probe (Tektronix P6015A) for voltage measurement and a current probe (LeCroy CP030) for current measurement. Optical emission spectroscopy (OES) data were collected using HR4000 instrumentation.

2.2. Properties of PAW

The detection of ROS/RNS was conducted utilizing the QuantiChrom™ NO assay kit and QuantiChrom™ peroxidase assay kit (BioAssay Systems, Hayward, CA). Total NO_x and H_2O_2 levels in the samples were quantified based on absorbance at 540 nm and 585 nm, respectively, using the BioTek Gen 5 microplate reader. The pH and electrical conductivity (EC) were measured using a Thermo Scientific Orion multifunction benchtop. Immediate measurements of EC and pH were taken post post-treatment. Furthermore, the oxidation-reduction potential (ORP) in deionized water (DI), PAW, and Mg-PAW was determined using an ExStik meter (Extech, model: RE300, China).

2.3. Seed germination and growth assay

Randomly selected Hordeum vulgare Perilla L. (barley) seeds were divided into three distinct groups, each comprising 120 seeds, and subjected to soaking under different conditions: DI water, PAW and Mg-PAW. The experiment was carried out in King Saud University, Saudi Arabia, and all agricultural practices were applied according to recommended national regulations for cultivated cultivar. Growth assays were initiated by planting seeds on sterile paper towels within plant culture dishes and soaked with DI water, PAW and Mg-PAW. Each culture dish accommodated 20 seeds, with experiments conducted in triplicate, resulting in a total of 120 seeds examined. The seeds were placed in plant culture dishes and then incubated in a growth chamber under controlled conditions at 25 ± 2 °C, 76 ± 5% relative humidity, and a 16/8-h light/dark cycle (44 W m^{-2} irradiance from a Philips Fluorescence Tube Light Bulb) until complete germination. Germinated seeds,

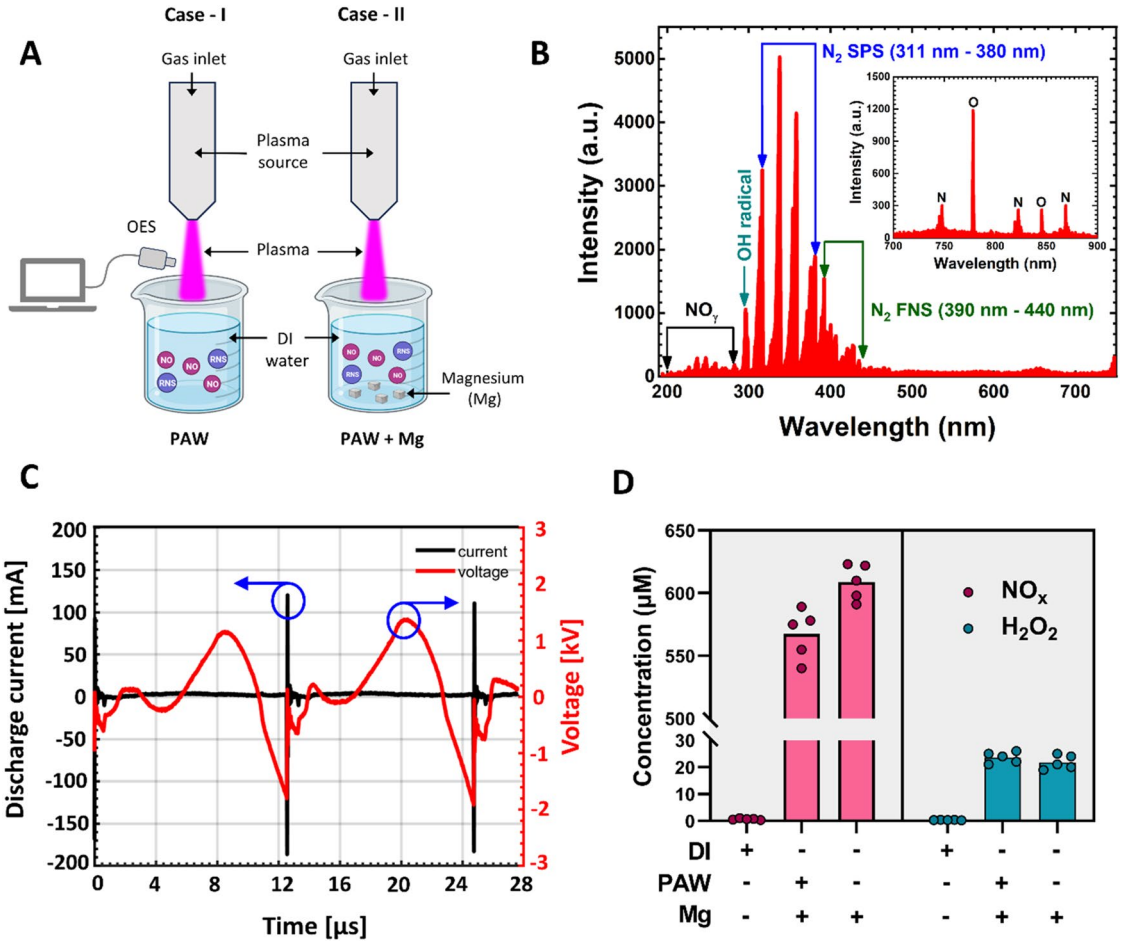

Figure 2. Experimental setup and properties of the device. (A) Scheme and arrangement to prepare PAW without Mg (Case I) and PAW with Mg or Mg-PAW (Case II). (B) OES spectra of air plasma. (C) Current and voltage waveforms. (D) The concentration of NO_x and H_2O_2 in the PAW and Mg-PAW. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

determined 24 h after sowing on plant culture plates, were computed using Equation (1) (Sivachandiran & Khacef, 2017):

$$\text{Germination rate} = \frac{\text{Number of germinated seed}}{\text{Total number of seeds}} \times 100\% \quad (1)$$

2.4. Determination of imbibition rate and growth assay of barley seeds seed water uptake

The assessment of water uptake capacity in barley seeds at distinct time intervals was conducted using a methodology explained in a referenced study (Bormashenko et al., 2015). Fifteen seeds were initially weighed (Wo) and immersed in DI, PAW and Mg-PAW. At various time points (1, 2, 3, 4, 6, 7, 11, 15, 20 and 24 h), the seeds underwent a gentle drying process with a paper towel to eliminate excess water, followed by reweighing (Wt). The percentage imbibition rate at each time point was computed using the subsequent Equation (2) as follows:

$$\text{IMB}(\%) = \frac{(Wt - Wo)}{\text{Wo}} \times 100 \quad (2)$$

For the investigation of plant growth, seeds were sown in pots filled with 30 g of vermiculite, and three replications were maintained for each experimental group (DI, PAW and Mg-PAW). Upon harvesting on the 10th day, measurements were taken for both root and shoot lengths. To determine plant dry weight, each experimental group underwent dehydration in a dry oven set at 65 °C for a period of five days or until a stabilized weight was achieved. Subsequently, the dry weight of each sample was recorded.

2.5. SEM analysis

The barley seeds were soaked with DI, PAW and Mg-PAW for 5 h and then analyzed for scanning electron microscope (SEM) analysis. The affixation of these seeds to a sample holder was achieved using electrically conductive double-stick carbon tape. Subsequently, an Omnifarious platinum coating process was applied with the assistance of an argon gas-powered Cressington 108 Auto Sputter coater. This process, executed for a duration of 20 s at a distance of 56 mm and a deposition rate of 0.33 Å/s, aimed to enhance sample conduction. The surface morphology of the seeds was examined using a field emission SEM (model: JEOL JSM-7001F).

2.6. Chlorophyll and carotenoid pigments detection

Fresh leaves, randomly harvested from ten barley plants, each weighing 200 mg, underwent meticulous dissection into fine pieces and then thoroughly homogenized with 5 mL of 80% acetone. The resulting mixture underwent vigorous inversion in test tubes. Subsequently, the test tubes assigned to both control and PAW-treated groups were shielded with aluminum foil and incubated at ambient temperature until the leaves achieved complete translucency. The filtrate was then utilized for absorbance measurements at three distinct wavelengths (470 nm, 646 nm and 663 nm) using a plate reader (Synergy HTX Multi-Mode Reader, Bio-Tek Instruments, Winooski, VT). These readings were employed to measure the chlorophyll and carotenoids. The entire pigment detection experiment was executed with a minimum of five replicates ($n = 5$).

2.7. Biochemical assays

2.7.1. Total soluble proteins (mg/g)

Fresh barley roots and leaves, each weighing 100 mg, underwent initial cleansing with distilled water, followed by immediate freezing with liquid nitrogen. Post-freezing, the samples were homogenized in 1 mL of PBS, and the resulting mixture underwent a 15-min centrifugation at 20,000 *g*. After decantation and transfer to another test tube, the isolated solution was subjected to the DC protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard to quantify total soluble protein (Bradford, 1976).

2.7.2. Malondialdehyde content (µmol/g FW)

The malondialdehyde (MDA) levels, indicative of membrane lipid peroxidation, were determined using the thiobarbituric acid (TBA) method. Leaves and root samples (300 mg) were homogenized in 3 mL of 0.1% trichloroacetic acid (TCA) using a precooled mortar and pestle. Following centrifugation at 10,000 × *g* for 10 min at 4 °C, the samples were incubated at 100 °C for 30 min with the addition of 1.0 mL of supernatant and 4.0 mL of TBA (0.67%) with 20% TCA. After rapid cooling, the fluorescence of the supernatant was measured using spectrophotometry (excitation at 540 nm and emission at 600 nm) in a 96-well black plate. At least five duplicate readings were taken for each sample (Zhang & Huang, 2013).

2.7.3. Proline content (µM/gm FW)

Barley leaves and roots (100 mg) were cryogenically ground, followed by homogenization with 5 mL of 3% sulphosalicylic acid solution. The resulting homogenate underwent centrifugation at 3000 × *g* for 20 minutes. A mixture of 2 mL glacial acetic acid and 2 mL acid ninhydrin was added to 2 mL of the supernatant, which was then boiled at 100 °C for one hour and cooled at room temperature. After blending with an equal volume of toluene, the mixture was spectrophotometrically measured for free toluene at 520 nm, using L-proline as the standard (Zhang et al., 2018).

2.7.4. Enzymes extraction and activity assays

A pre-cooled mortar and pestle containing 4.5 ml of PBS buffer (0.1 M, pH 7–7.4) was used to cryogenically grind 500 mg of fresh leaves. For 20 min at 4 °C, the homogenate was centrifuged at 15,000 rpm. Later enzyme tests were conducted using the supernatant.

2.7.5. Ascorbate content (µg/mg protein)

The Elabscience Vitamin C Test Kit (Houston, TX) was employed for colorimetric measurement of ascorbic acid in barley leaves. The kit facilitated a two-stage color reaction: first, Ascorbic acid converted Fe^{3+} to $Fe^{2+,}$ and second, Fe^{2+} interacted with phenanthroline, resulting in color formation. The absorbance of the supernatant was measured at 536 nm using a microplate reader (Synergy HTX Multi-Mode Reader, Bio-Tek Instruments, Winooski, VT).

2.7.6. Superoxide dismutase (SOD) assay

SOD activity in barley leaves was assessed using the Elabascience Assay Kit (Houston, TX). The kit employed xanthine oxidase (hydroxylamine technique) for SOD

activity testing. The procedure involved adding 20 µL of the sample supernatant to the detecting reagent, thoroughly mixing and incubation for 20 min at 37 °C. SOD activity was measured at 450 nm using a microplate reader.

2.7.7. Catalase activity assay (U/L)

Catalase (CAT) activity in barley leaves was assessed using the Enzyme ChromTM Catalase Assay Kit (ECAT-100, Bio-assay System), following the manufacturer's guidelines. One unit of CAT activity was defined as the amount needed to decompose 1 µmol of H_2O_2 per minute at room temperature and a pH of 7. The activity was measured at 570 nm and expressed in U/L.

2.8. Statistical analysis

The data derived from three independent experiments ($n = 3$) underwent comprehensive analysis and were presented as the mean ± standard error, utilizing Microsoft Excel software (Microsoft Office 365) and GraphPad Prism. To ascertain the statistical significance of the outcomes, the student's t-test was employed. Significance levels were determined by considering p values less than 0.05 as indicative of statistical significance. The representation of statistical significance was denoted using asterisks, where $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3. Results

3.1. Characteristics of CAP source

The experimental setup for the preparation of PAW and Mg-PAW. The DI water was directly exposed to CAP without magnesium (PAW) and with magnesium (Mg-PAW) as shown in Figure 2(A). The OES spectra of the generated plasma are given in Figure 2(B). Within the UV-C range (200–280 nm wavelength), discernible emissions in the $NO\gamma$ bands are identified with relatively weak signals. These species are thought to have arisen from the reaction of energetic electrons with ambient air molecules of N_2 and O_2, which act as the precursor gas. The N_2 second positive system (SPS) was observed between 311 and 380 nm. Moreover, emissions from the nitrogen first negative system (FNS) can be seen in the UV-A (390–440 nm) and visible spectrums. Furthermore, strong emissions are produced by the dissociation of oxygen molecules, especially from atomic oxygen at 777 nm (Figure 2(B)). The current-voltage waveforms are provided in Figure 2(C).

The concentration of NO_x and H_2O_2 was measured and presented in Figure 2(D). The NO_x concentration was measured as 0.66, 567.4 and 608.8 µM in DI, PAW and Mg-PAW, respectively. Similarly, the H_2O_2 concentration was measured as 0.36, 23.6 and 21.8 µM in DI, PAW and Mg-PAW, respectively. The pH level of the irrigated water underwent measurement, revealing a pH of 6.09 for DI water. Subsequently, the pH of PAW decreased to 4.02. Notably, the introduction of Mg resulted in the neutralization of the pH in Mg-PAW, elevating it to 6.4. These results indicate the role of Mg in effectively neutralizing the pH levels in the treated water (Figure 3(A)). The ORP values for DI water, PAW and Mg-PAW were quantified at 152.75 mV, 426.75 mV and 395.75 mV, respectively (Figure 3(B)). Similarly, the EC measurements under specified conditions yielded values of 23 µs/cm for DI water, 97.15 µs/cm for PAW and 85.75 µs/cm for Mg-PAW (Figure 3(C)).

3.2. Enhanced seed germination and seedling growth by Mg-PAW in barely

The barely seeds irrigated with PAW and Mg-PAW resulted in a remarkably higher seed germination rate, with the uppermost germination rate noted in the Mg-PAW. Figure 4(A) presents photographs of barley seed progression during the germination stages on days 1, 3 and 5. Correspondingly, Figure 4(B) illustrates the temporal dynamics of germination rates. Notably, it is observed that, in Mg-PAW, the germination rate exhibited a 2.29-fold increase on day 2 and a 2.59-fold increase on day 3 in comparison with the seeds irrigated with DI water. Furthermore, noteworthy observations indicate that the incorporation of Mg in Mg-PAW, aimed at pH neutralization, results in elevated germination rates as compared to the utilization of PAW alone (Figure 4(B)).

The photographs and SEM images of barley seeds taken one day following irrigation with DI water, PAW and NO-PAW and shown in Figure 4(C). The images indicate a clear augmentation in seed size after water absorption, particularly in the PAW and Mg-PAW groups compared to DI water. The SEM observations showed that none of the three conditions (DI water, PAW and Mg-PAW) induced significant alterations in the surface morphology of the seeds (Figure 4(C)). Figure 4(D) presents the percentage of water uptake by barley seeds following irrigation with DI water, PAW and Mg-PAW. Remarkably, both the PAW and Mg-PAW groups exhibit elevated

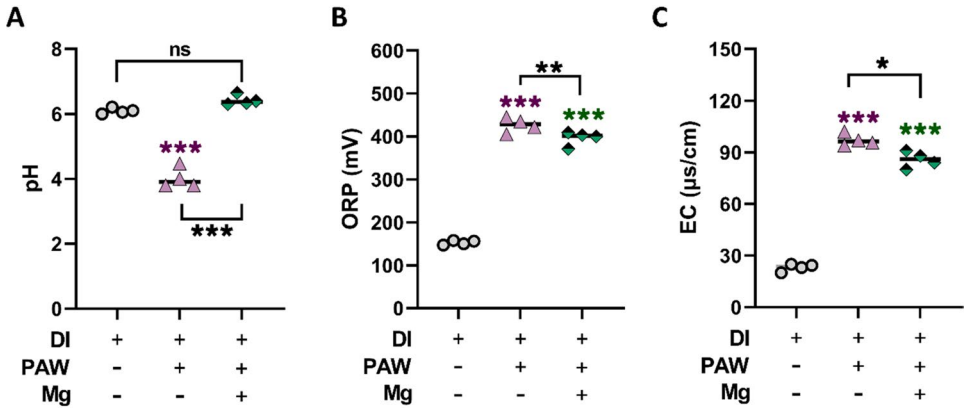

Figure 3. The characteristics of water used for irrigation, include DI water, PAW and Mg-PAW. (A) pH. (B) ORP. (C) EC. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

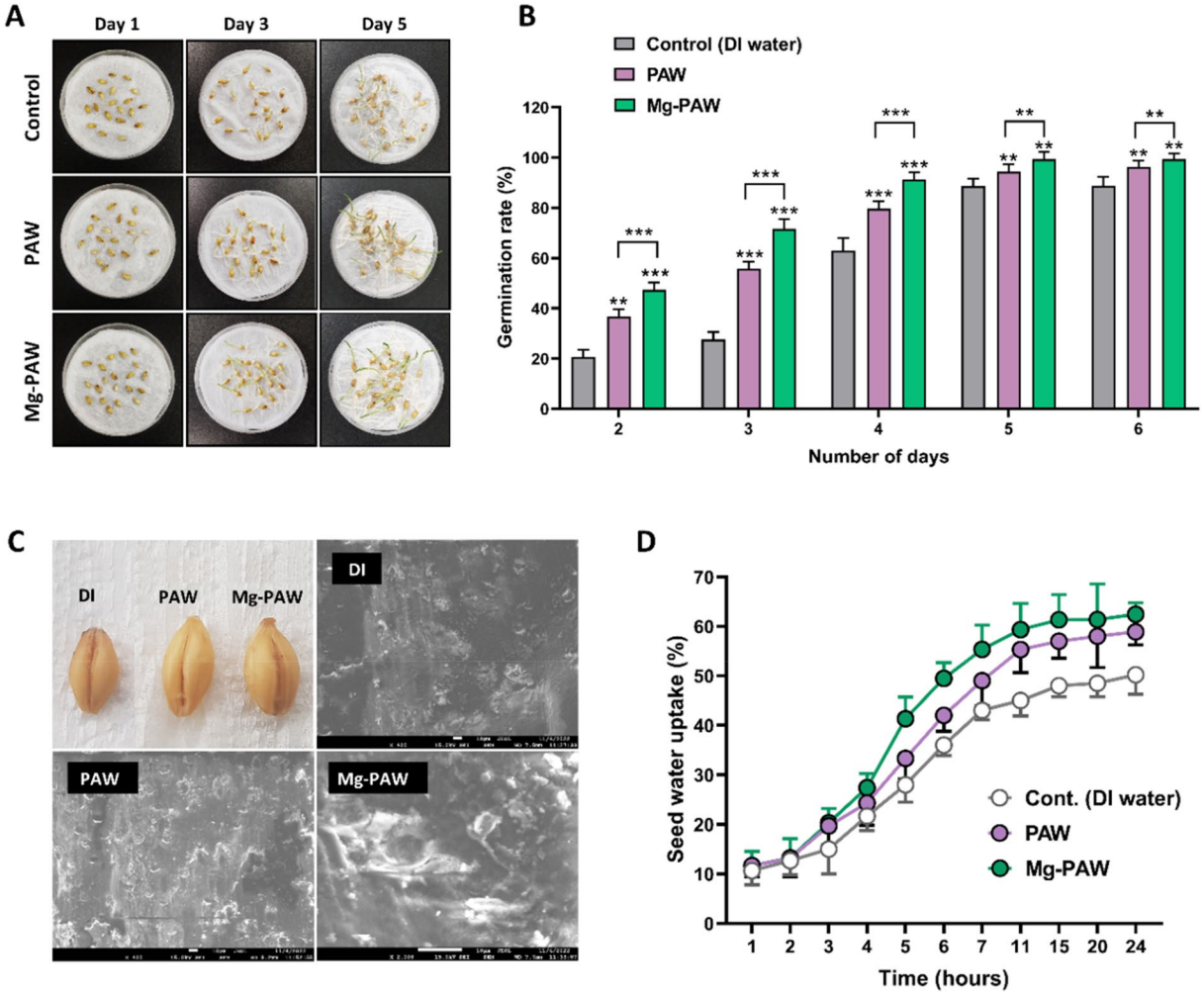

Figure 4. Germination rate and seed water uptake. (A) Photographs of the seed show the development of seed germination at three different time intervals: day 1, day 3 and day 5. (B) Seed germination rate in DI, PAW and Mg-PAW. (C) Photographs of seeds treated in three different conditions (DI, PAW and Mg-PAW). SEM images of barley seeds indicated no observed changes on the seed surface in selected treatment conditions. (D) Seed water uptake percentage. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

levels of water uptake in comparison to the control group (DI water).

Photographs illustrating different seedling stages of barley are presented in Figure 5(A–C). Figure 5(C) shows the photograph of barley on day 10, corresponding to the harvesting period. The length of the root and shoot was measured on day 10 in all irrigation conditions. The root length was measured as 6 cm in DI water, 11.17 cm in PAW and 16.33 cm in Mg-PAW (Figure 5(D)). Notably, the root length exhibited a 1.86-fold increase in the PAW treatment compared to DI water, while Mg-PAW demonstrated a significant 2.72-fold increase compared to DI water. Interestingly, when compared to PAW alone, Mg-PAW, featuring neutralized pH, exhibited a 1.46-fold higher root length. Similarly, the root length was measured as 6.88 cm in DI water, 9.25 cm in PAW and 13.33 cm in Mg-PAW (Figure 5(E)). The shoot length exhibited a 1.34-fold increase in the PAW treatment, while Mg-PAW demonstrated a significant 1.93-fold increase compared to DI water. Interestingly, Mg-PAW with neutralized pH showed a 1.44 times longer shoot length than PAW alone.

The plant's fresh weight was measured as 0.28, 0.39 and 0.46 grams in DI water, PAW and Mg-PAW. Compared to the control (DI water), the plant irrigated with PAW, and Mg-PAW shows a 1.39-fold and 1.64-fold increase, respectively. Furthermore, the Mg-PAW shows a 1.17-fold increase in fresh weight compared to PAW (Figure 5(F)). Similarly, when the plant's dry weight was measured, it was found that the PAW-irrigated plant and the Mg-PAW-treated plant had increases of 1.5 and 1.79 times, respectively, when compared to the control (DI water). In addition, the dry weight of the Mg-PAW is 1.19 times higher than that of the PAW (Figure 5(F)).

3.3. Biochemical profiling of oxidative stress markers in barley seedlings

Barley seedling biochemical profiling was conducted to assess the impact of oxidative stress induced by PAW. The photosynthetic efficiency of seedlings, directly associated with chlorophyll content in leaves, was examined. Notably, both the PAW and Mg-PAW treatment groups exhibited significantly higher

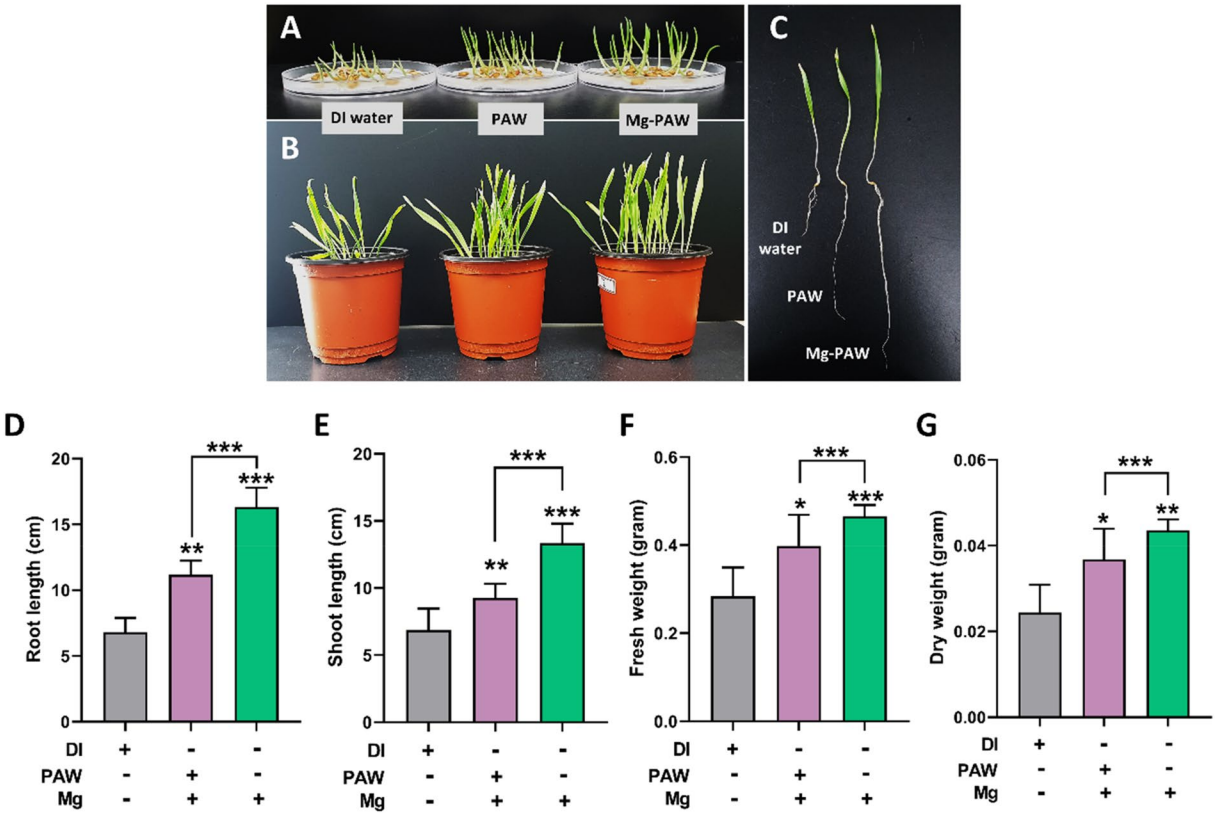

Figure 5. Growth parameters. (A) The photographs of barley at the germination stage. (B) The photographs of the barley plant when grown in vermiculite on day 7. (C) The photograph at day 10 after harvesting. (D) Rooth length. (E) Shoot length. (F) Plant fresh weight. (G) Plant dry weight. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

chlorophyll content compared to the control group (DI water), as illustrated in Figure 6(A–C). Specifically, the total chlorophyll content in barley seedlings irrigated with PAW demonstrated a substantial 2.61-fold increase relative to DI water. Furthermore, the Mg-PAW group displayed a remarkable 3.13-fold elevation in chlorophyll content compared to DI water, and a noteworthy 1.2-fold increase compared to the PAW group.

A significant elevation in carotenoid levels in both the PAW and Mg-PAW groups (Figure 6(D)). The barley irrigated with DI water displayed carotenoid levels of 295.57 mg/g however it significantly increased to 612.41 mg/g in PAW and 729.69 mg/g in the Mg-PAW group. Furthermore, the total soluble protein was also observed in the leaf and root of barley, and results are presented in Figure 6(E,F). The total soluble protein levels in the leaf increased 2.3-fold in PAW and 2.76-fold increase in the Mg-PAW group compared to the group irrigated with DI water. Interestingly, the Mg-PAW shows 1.2-fold higher total soluble protein levels in the leaf compared to PAW alone. Similarly, the root's total soluble protein levels exhibited a 1.6-fold increase in response to PAW and a noteworthy 3-fold elevation in the Mg-PAW group, in comparison to the DI water irrigated group. Interestingly, Mg-PAW displayed a 1.8-fold higher total soluble protein level in the root compared to PAW alone.

Biochemical profiling of barley seedlings was performed to explore the effect of oxidative stress induced by PAW and Mg-PAW. MDA is a prominent

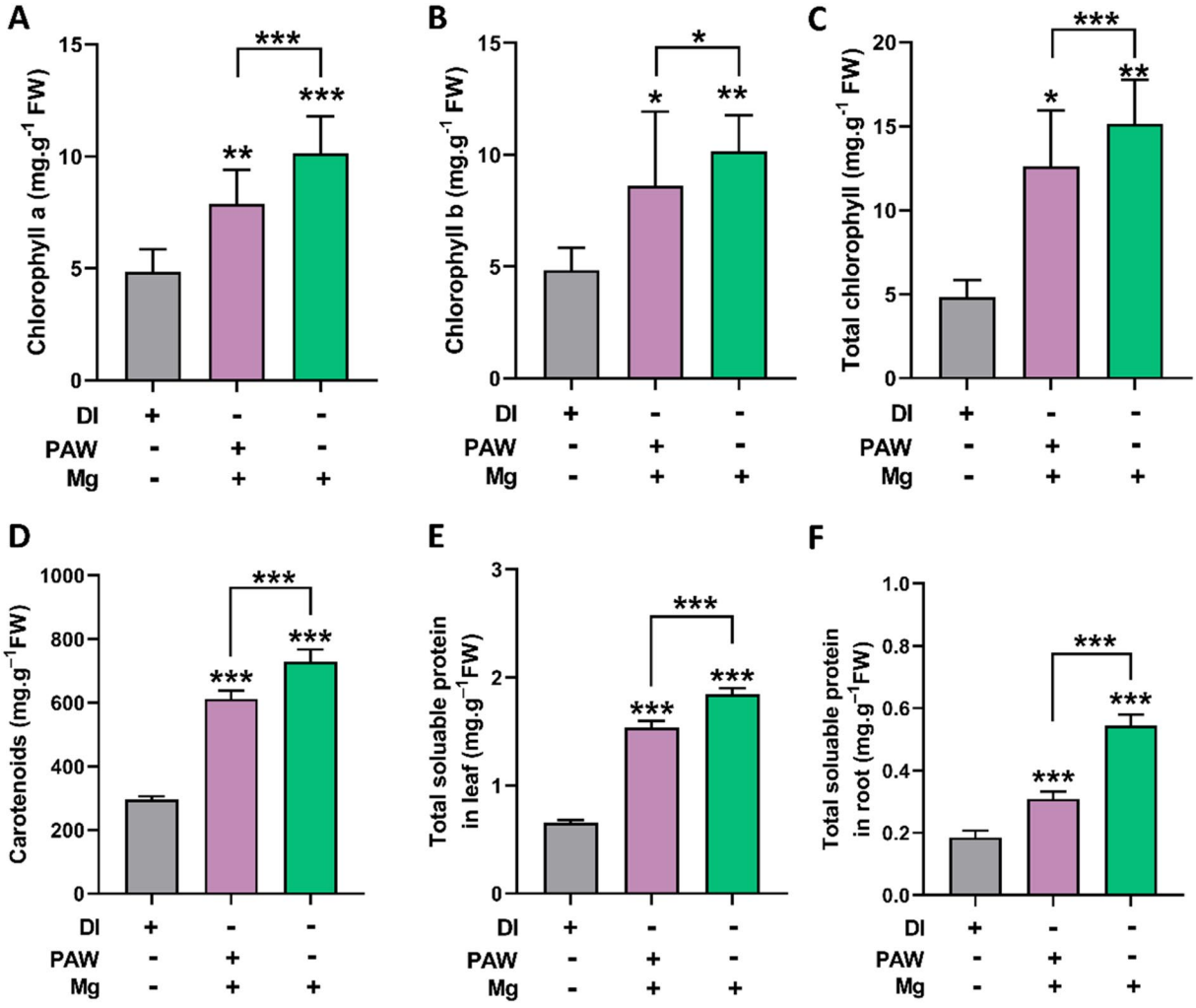

Figure 6. Estimation of biochemicals in barley seedlings. (A–C) Chlorophyll: enhanced chlorophyll levels in barley seedlings in PAW and Mg-PAW groups. (D) Carotenoids: Elevated carotenoid levels were observed in PAW and Mg-PAW, with the most pronounced increase in Mg-PAW condition. (E,F) Total soluble protein (leaf and root): higher protein content in leaf and root tissues following PAW and Mg-PAW treatment. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

stress marker that provides information regarding the membrane damage caused by reactive species produced by PAW and Mg-PAW. The MDA levels were measured in the leaf (Figure 7(A)) and root (Figure 7(B)) of barley irrigated with DI water, PAW and Mg-PAW. It is noted that the MDA content significantly reduced when irrigated with PAW and Mg-PAW while the highest reduction was observed in Mg-PAW when pH is neutralized by using Mg. The leaf's MDA content exhibited a reduction of 40% and 58% in PAW and Mg-PAW groups, respectively, compared to the group irrigated with DI water. Furthermore, in the comparison between the PAW and Mg-PAW groups, the Mg-PAW demonstrated a 30% higher reduction in MDA content than PAW alone. Similarly, in the root, MDA content experienced reductions of 38% and 66% in PAW and Mg-PAW, respectively, as compared to the group irrigated with DI water. Notably, when contrasting the PAW and Mg-PAW groups in barley roots, Mg-PAW exhibited a 46% higher reduction in MDA content than PAW alone.

Under the influence of stress signals, the osmoprotectant amino acid proline undergoes upregulation, serving as a crucial mechanism to mitigate the accumulation of excessive hydroxyl radicals. This regulatory response contributes to the preservation of the plant's osmotic potential while concurrently shielding enzymes from oxidative damage. When compared to the control (DI water) group, the proline content in seedlings irrigated with PAW increased 1.3-fold and 1.73-fold with Mg-PAW. Furthermore, the Mg-PAW exhibits a 1.3-fold higher increase in proline in the leaf than PAW alone (Figure 7(C)). Similarly, both PAW and Mg-PAW groups show a 1.5-fold increase in proline in root compared to the group irrigated with DI water (Figure 7(D)).

The H_2O_2 within cells can be successfully neutralized by the well-known small antioxidant ascorbate. The ascorbate concentration displayed reductions of

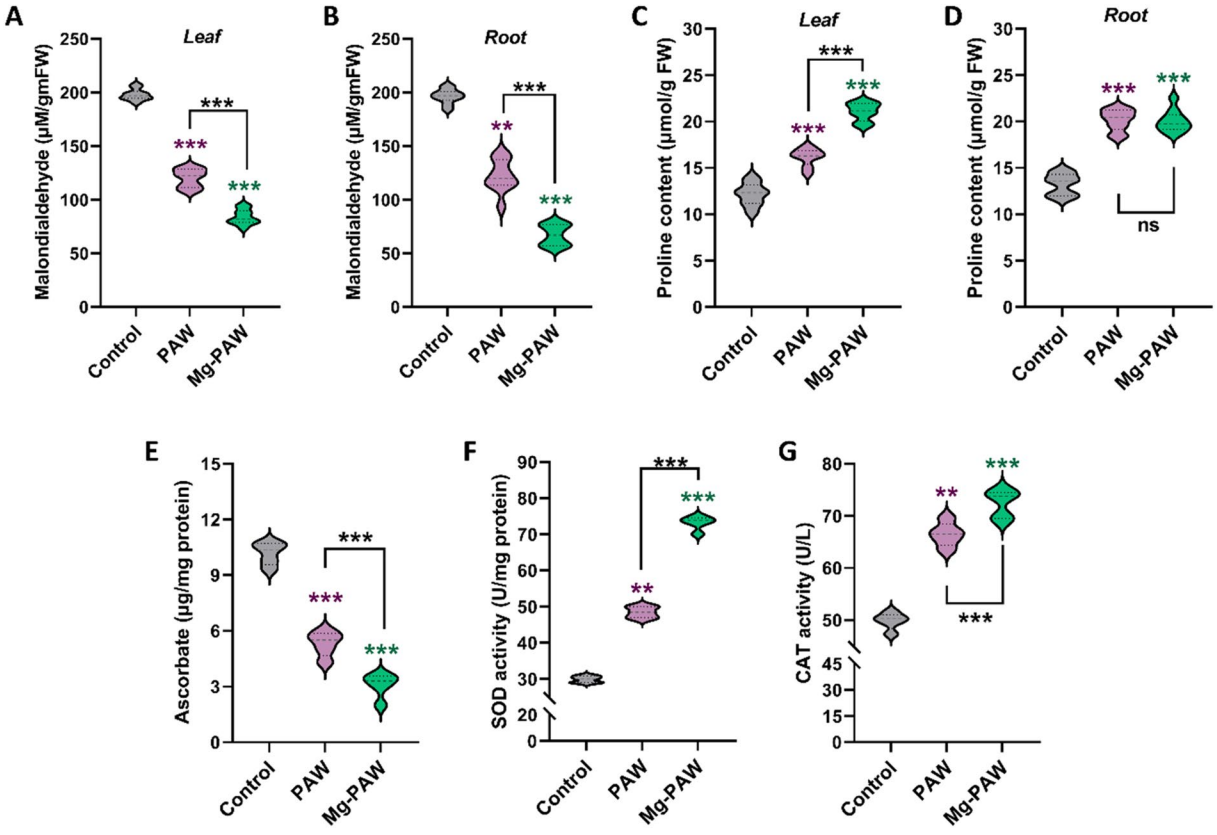

Figure 7. Estimation of enzymatic activities in barley seedlings. (A,B) MDA content in leaf and root of barley: significant reduction in MDA content in PAW, and Mg-PAW groups compared to controls (DI water). (C,D) Proline content in leaf and root of barley: higher proline content with more than a 2-fold increase observed in PAW, and Mg-PAW groups. (E) Ascorbate concentration: application of PAW and Mg-PAW significantly reduced the ascorbate concentration in barley seedlings. (F) SOD activity: SOD activity significantly increased, with the highest levels recorded in the Mg-PAW group. (G) CAT enzyme activity: higher CAT enzyme activity was observed in the Mg-PAW group. Microsoft Excel software (MS Office 365) was utilized for determining statistical significance ($n = 3$). Significance among treatment groups was elucidated by asterisks, with significance levels specified as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

48% and 70% in barley seedlings when irrigated with PAW and Mg-PAW, respectively (Figure 7(E)). The noted reduction in ascorbate concentration signifies an augmented capability for the detoxification of ROS. This pattern is similar to the SOD activity, with an increase of 1.6-fold in PAW and 2.44-fold increase in Mg-PAW in comparison to the control (DI water) group (Figure 7(F)). The Mg-PAW groups showed a 1.5-fold higher SOD level compared to PAW alone. Moreover, the heightened CAT enzyme activity observed in barley seedlings subjected to irrigation with PAW and Mg-PAW signifies an enhanced antioxidative response in the plant. Through the activation of this defense mechanism, the plant can effectively mitigate potential damage to cellular components and neutralize any excessive amount of H_2O_2. CAT activity in barley seedlings displayed a notable 33% increase in PAW and 45% in the Mg-PAW group compared to control (Figure 7(G)). Furthermore, neutralizing the pH of PAW using Mg provides 8% higher CAT activity observed in the Mg-PAW group (Figure 7(G)).

4. Discussions

The treatment of water with CAP to produce PAW has a variety of applications in the biological field particularly in agriculture (Adamovich et al., 2022; Ito et al., 2012; Wang et al., 2023). We aimed to investigate the effect of PAW with low pH and Mg-PAW with neutralized pH on the seedling growth of barley. The use of Mg while producing PAW successfully neutralized the pH by removing the H^+ ion. In this way, the removal of H^+ ions enables their transformation into nascent hydrogen and eventually neutralizes the pH of PAW. Additionally, the introduction of metal ions, such as Mg, into the water matrix elicits positive physiological modifications within plant biology (Song et al., 2023). This process indicates that the existence of Mgions may provide a positive influence on important physiological processes, thus enhancing various facets in plants for better growth and development.

Within the discharge region, the collision of electrons induces the excitation (Peng et al., 2018) or dissociation (Penetrante et al., 1996) of the N≡N molecule. Excited nitrogen molecules may dissociate further when additional plasma energy is provided. The availability of RNS influences several physiological processes of plants. So, it is important to discuss the formation mechanism of RNS using CAP. The RNS after CAP discharge can be induced as follows:

$$N_2 + e \rightarrow 2N + e \quad (3)$$

$$N_2 + e \rightarrow {\bullet}N_2 + e \quad (4)$$

$${\bullet}N_2 + e \rightarrow 2N + e \quad (5)$$

When the OH radicals exist, atomic nitrogen experiences oxidation, subsequent in the formation of highly reactive •NO. Predominantly, the formation of NO_x species in PAW can be explained using equations as follows.

$$N + {\bullet}OH \rightarrow NO + H \quad (6)$$

$$NO + OH \rightarrow NO_2 + H \quad (7)$$

$$NO_2 + OH \rightarrow NO_3 + H \quad (8)$$

The further dissociation of NO_x forms NO_2^-, NO_3^- and H^+ in the liquid (aqueous) medium. In this work, the use of Mg played a key role in the conversion of NO_2^- and NO_3^- into Mg nitrates $Mg(NO_2)_2$ and nitrites $Mg(NO_3)_2$ (Wenjuan & Xiangli, 2007). This process includes the combination of Mg ions into the nitrogen-containing compounds of PAW. It is noteworthy that metal nitrates and nitrites, formed through this mechanism, manifest as neutral salts (Equations (9) and (10)).

$$Mg^{+++} + NO_2^- \rightleftharpoons Mg(NO_2)_2 \quad (9)$$

$$Mg^{+++} + NO_3^- \rightleftharpoons Mg(NO_3)_2 \quad (10)$$

Electrons derived from the metal initiate the reduction of H^+ to H (Kumari et al., 2018). The inclusion of Mg in PAW plays a substantive role in elevating the pH value (expressed as $=-\log[H^+]$), owing to the effective reduction of H^+. This phenomenon stems from the metal's capacity to facilitate electron transfer reactions, resulting in a notable increase in the concentration of H, thus influencing the overall pH of the PAW. In our findings, it was observed that immersion of Mg led to a significant increase in pH, ultimately reaching levels comparable to those observed in DI water, as depicted in Figure 3(A). It is well known that PAW is useful for plant growth and development. However, pH is also a key factor for plant growth. Plasma treatment can reduce the pH of PAW between 4 and 5 which limits its applications and unable to harness its maximum benefit for plant growth. To harness the maximum advantage of PAW, Mg played a key role in removing the acidic nature of PAW by keeping the ROS/RNS inside Mg-PAW. This is why, Mg-PAW with neutralized pH provided more advantageous effects compared to PAW alone.

In previous studies, PAW has been used to enhance seed germination and plant growth (Sivachandiran & Khacef, 2017; Guragain, Baniya, et al., 2021; Guragain, Pradhan, et al., 2021; Darmanin et al., 2020). PAW contains various ROS/RNS which can have both beneficial and detrimental effects, depending on their concentration levels. Up to some specifically optimized concentration, the ROS/RNS in PAW produces beneficial effects to promote germination and plant growth (Che et al., 2024; Tikekar & Jha, 2024). Typically, PAW is generated with an initial low pH, necessitating neutralization to uphold the sustainability of plasma agriculture. The use of acidic water for irrigation has been identified as detrimental, inducing adverse effects, such as root burns in plants. Prolonged exposure to such acidic PAW can further result in complete infertility of the soil. Notably, in the course of nitrogen oxidation processes, the pH of PAW was observed to decrease significantly, reaching levels as low as 2 (Sivachandiran & Khacef, 2017; Judée et al., 2018; Adhikari et al., 2020). The incorporation of Mg to produce Mg-PAW imparts supplementary advantages for agricultural applications. Both PAW and Mg-PAW exhibit higher germination rates and growth in barley seedlings compared to DI water (control). Nevertheless, upon a comparison between PAW and Mg-PAW, the germination rate and seedling growth of barley manifest higher levels in Mg-PAW, where the pH is neutralized. These effects were attributed to the neutralization of Mg-PAW, which optimizes the environmental conditions for barley seedling development along with the availability of reactive species produced by CAP. The neutralized pH in Mg-PAW contributes to a more favorable physiological environment, fostering enhanced germination and subsequent growth in comparison to the non-neutralized PAW (Figures 4 and 5).

The chlorophyll content and carotenoid levels exhibited a notable increase in both PAW and Mg-PAW treatments in barley seedlings (Figure 6). This enhancement suggests a positive impact on the photosynthetic pigments crucial for plant development (Lisiewska et al., 2006). The PAW with numerous ROS/RNS can play a key role in signaling bio-molecules. These ROS/RNS associated with PAW are involved in activating various signaling pathways that are linked to chlorophyll and carotenoid biosynthesis in plants, leading to increased pigment concentrations (Than et al., 2022). It is well known that Mg is essential for chlorophyll synthesis in plants which facilitates the nutrient absorption which are essential for the formation of this pigment (Willows, 2019). In this experiment, higher chlorophyll content and carotenoid pigment concentrations were detected after PAW and Mg-PAW irrigation to barley seedlings (Siddiqui et al., 2019; Moharekar et al., 2003). The Mg ions are vital macronutrients for plants to support several physiological developments including various enzyme activities, photosynthetic activities, molecular gene activities linked to plant growth, gene suppression associated with growth inhibition and reproduction (Ahmed et al., 2023). Furthermore, other base substances might also be capable of neutralizing the pH of PAW by eliminating H^+ ions. However, it is important to understand that the use of strong bases can actually alter the soil pH.

The decreased levels of MDA content and increased proline levels indicate that PAW and Mg-PAW irrigated groups produced improved antioxidant processes in barley seedlings (Figure 7(A–D)). These results highlight the better capacity of the barley seedlings to mitigate oxidative stress, signifying a strong defense against lipid peroxidation in PAW and Mg-PAW groups. The decreased MDA levels indicate a more strong antioxidant defense system in barley seedlings. However, the elevated proline content indicates a possible role in osmotic regulation leading to total cellular resilience (Figure 7(A–D)). Correspondingly, the ascorbate content reduction after PAW and Mg-PAW irrigation suggest increased ascorbate peroxidase and ascorbate oxidase activity (Figure 7(E)) (Yoshimura et al., 2000). H_2O_2 and NO function as signaling molecules, prompting cellular growth and activating defense/detoxification systems, while superoxide exerts detrimental effects on cellular integrity (del Río, 2015; Neill et al., 2002). In this study, the upregulation of reactive species induces the antioxidant machinery in seedlings, contributing to the maintenance of redox homeostasis. Elevated SOD activity, particularly observed in PAW and Mg-PAW, signifies an augmented conversion of superoxide radicals into H_2O_2 (Figure 7(F)). Notably, superoxide radicals play a pivotal role in growth-associated processes such as root development, cell wall expansion, growth hormone production, pollen tube growth and endosperm development. Furthermore, an enhancement in CAT activity was noted in groups irrigated with PAW and Mg-PAW in comparison to the control (DI water) (Figure 7(G)). This observation suggests that CAP may play a pivotal role in redox regulation by influencing the antioxidant enzyme activity. The improved CAT activity implies an augmented capacity of the plant system to decompose H_2O_2, reinforcing the

contention that CAP treatments contribute significantly to the redox balance within the biological system.

5. Conclusions

Utilization of CAP technology has the potential to decrease water pH, potentially constraining the utility of PAW in applications involving pH-sensitive plants. In this study, Mg was employed to mitigate the acidity of PAW by reducing the H^+ ions. In this study, we assessed the impact of Mg addition to PAW (Mg-PAW), a potential mitigator of water acidity, on barley germination and growth, compared to CAP technology without Mg (PAW). Results showed higher seed germination in both treatment groups, with better results following Mg-PAW application. The total chlorophyll content in barley seedlings irrigated with PAW and Mg-PAW was 2.61 and 3.13 times higher than similar measurements following DI water. Additionally, PAW and Mg-PAW applications increased carotenoid content by 107% and 147% compared to DI water. Moreover, and compared to PAW alone, Mg-PAW application increased total soluble protein level, SOD activity, and CAT activity more than PAW alone, while also further decreasing the MDA content compared to PAW application. The implications of these findings carry significant relevance in the realm of agricultural applications, presenting a promising prospect for enhanced plant growth through the utilization of Mg-PAW with neutralized pH. This study contributes to the advancement of our comprehension of plant responses to PAW and Mg-PAW, thereby offering a potential solution to the global imperative for sustainable crop production.

Informed consent statement

Not applicable.

Author contributions

Conceptualization, M.F.S. and B.A.A.; methodology, M.F.S. and B.A.A.; software, M.F.S. and B.A.A.; validation, M.F.S., N.A. and B.A.A.; formal analysis, M.F.S., E.Z.N., H.I.G. and B.A.A.; investigation, M.F.S. and B.A.A.; resources, M.F.S. and B.A.A.; data curation, E.Z.N., M.L.B., N.A. and B.A.A.; writing – original draft preparation, M.F.S. and B.A.A.; writing – review and editing, M.L.B., N.A. and H.I.G. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

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Data availability statement

Data are available from the corresponding author upon reasonable request.

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