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Cold Atmospheric Plasma-Activated Water Irrigation Induces Defense Hormone and Gene expression in Tomato seedlings

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Plants are very vulnerable to pathogen attacks and environmental stress as they are exposed to harsh environments in natural conditions. However, they have evolved a self-defense system whereby reactive oxygen and nitrogen species (RONS) act as double-edged swords by imposing (at higher concentration) and mitigating (at lower concentration) environmental stress. Cold plasma is emerging as a feasible option to produce a variety of RONS in a controlled manner when amalgamate with water. Cold plasma activated/treated water (PAW) contains a variety of RONS at concentrations, which may help to activate the plant's defense system components. In the present study, we examine the effect of cold atmospheric-air jet plasma exposure (15 min, 30 min, and 60 min) on the water's RONS level, as well as the impact of PAW irrigation, (assigned as 15PAW, 30PAW, and 60PAW) on tomato seedlings growth and defense response. We found that PAW irrigation (priming) upregulate seedlings growth, endogenous RONS, defense hormone (salicylic acid and jasmonic acid), and expression of key pathogenesis related (PR) gene. 30 min PAW contains RONS at concentrations which can induce non-toxic signaling. The present study suggests that PAW irrigation can be beneficial for agriculture as it modulates plant growth as well as immune response components.

Plants, which are immobilized organisms, often face many challenges like harsh environments, pathogens and herbivores attacks. In response to abiotic and biotic stresses, plants have evolved an adaptive defense system for survival¹. Reactive oxygen and nitrogen species (RONS) are byproducts of cellular metabolism and play a binary role in aerobic life, depending on their concentration². RONS in small concentrations act as signaling molecules and regulate cellular growth, development, and defense processes. However, at very high concentrations they are deleterious to cellular biomolecules and lead to cell death³. Hydrogen peroxide (H₂O₂) and nitric oxide (NO) are the most studied and well known RONS in aerobic systems. H₂O₂ is the most stable and strong oxidant molecule and initiates the production of various other reactive oxygen radicals, such as superoxide, hydroxyl, and NO_x, in cells. H₂O₂ predominantly generates as a byproduct of photorespiration and photosynthesis in chloroplasts and mitochondria. The H₂O₂ acts as a vital signaling molecule and stimulates the production of other signaling molecules such as Ca²⁺, mitogen activated protein kinase (MAPK), hormone-like salicylic acid (SA), jasmonic acid (JA), abscisic acid, and ethylene⁴. NO is another interesting signaling molecule, which has attracted a lot of attention due to its multifarious role in plant growth and development. NO transduces the environmental signals by cAMP, altering the Ca²⁺ concentration in the cytosol, and interacts with H₂O₂. NO regulates the gene expression of various antioxidant enzymes, pathogenesis related (PR) proteins, and MAPK⁵. H₂O₂ and NO signaling molecules exhibit a cross-talk and both synergistic and antagonistic interactions with each other⁶.

Both biotic and abiotic stresses cause RONS bursts, which induce the production of secondary metabolites as well as defense hormones precursors in plant cells. When a pathogen invades the host system, the plant endogenous RONS level increase, which triggers defense genes expressions, such as SA and JA synthesis pathway genes and PR genes. These genes kill the pathogen and prevent it from infecting other cells by the hypersensitive

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response and systemic acquired resistance (SAR)⁷. H₂O₂ and NO mutually modulate phytohormone synthesis in plant cells and activate SAR by inducing the concentration of SA, JA, ethylene, and brassinosteroid^{8,9}.

The JA synthesizes in plant cells in response to wounding, herbivorous attacks, and necrotropic pathogen infections. It also mediates RONS bursts and activates the hypersensitive response¹⁰. JA levels are modulated via NO concentration in plant cells. Bioinformatics analysis of NO responsive genes and promoters revealed that JA synthesizes genes such as oxophytodieneic acid reductase and upregulates expression in response to NO elicitors in *Arabidopsis thaliana*⁹. SA is a derivative of phenol synthesized from chorismate by isochorismate synthase. It is amalgamated in response to pathogen infections and develops local and systematic resistance¹¹. As well as JA, plants have another defense phytohormone, synthesized via octadecanoid pathways using linolenic acid as a precursor. JA promotes the synthesis of flavonoids and other phenolic compounds¹². SA and JA both regulate plant vegetative growth, seed germination, root growth, nutrient uptake, water relations, Ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity, chlorophyll content, and stomata closure^{13,14}. They also affect the redox homeostasis by regulating the activities of antioxidant enzymes such as peroxidase, polyphenol oxidase, superoxide dismutase (SOD), catalase (CAT), and phenylalanine ammonia lyase (PAL)^{15,16}. The upregulation of SA and JA in plants can enhance both their pathogen resistance potential and their growth¹³.

Genetic engineering approaches have frequently been attempted to improve plant growth and defense quality^{17,18}. However, concerns for genetically modified organisms and their products have limited the general use of this technology¹⁹. Hence in the present scenario, the priming of plants is most extensive research topic in area of plant protection. Priming is a physiological process to achieve the readiness status of plants against biotic and abiotic stress²⁰. Primed plants have shown a vigorous and faster response against stress²¹. Priming was done to boost plant immunity against pathogens and stress. Small biomolecule and stimuli used for induced immune response are called immune inducer. Various natural and chemical (an analog of the natural compound) inducers have been reported²². Cold plasma and cold plasma treated liquids are also used to induce plant growth.

Cold atmospheric pressure plasma (CAP) has attracted a lot of attention from plant biologists from the past few years due to its potential for enhancing plant growth and defense. CAP technology is a harmless, sensitive, ecofriendly and economical approach to improve plant health as well as crop production²³. CAP is an ionized gas consisting of a mixture of radicals, UV rays, and electromagnetic waves, which initiates the production of various RONS in micro-environments^{24,25}. CAP treatment to water modifies its chemical property by converting water into a RONS cocktail. RONS have a significant impact on plant metabolism and plant growth and development². H₂O₂ and NO, small signaling biomolecules, are produced endogenously in different cellular metabolism processes and regulate plant responses to various abiotic and biotic stresses²⁶.

The previously cited literature on the regulatory role of RONS (especially H₂O₂ and NO) in cellular metabolism, growth, and defense motivates us to investigate the impact of cold plasma generated H₂O₂ and NO in the water on plant defense transcripts and phytohormone. Therefore, in this study we investigate the effect of different cold PAW irrigations on the growth of seedlings, endogenous RONS concentration, oxidative toxicity, defense related (antioxidant, PR, and hormone) gene expression, and defense hormone content in seedlings. The results of this study indicate that PAW is acting as an inducer for growth as well as defense related components. To our best knowledge, it is the first study which shows that PAW treatment induces defense hormone production as well as other transcripts in seedlings and acts as an immune inducer. On the basis of the present study's results, a schematic diagram was prepared to show the biochemical and molecular events occurring in plant cells in response to PAW-induced RONS burst.

Results

We observed changes at physiological, biochemical and molecular level in tomato seedlings treated with PAW as compared to control seedling. The diagrammatic representation of the experiment strategy is shown in Fig. 1a. The air plasma jet instrument used in this study was developed in Plasma Research Bioscience Center, Kwangwoon University, Seoul, South Korea. The experimental setup of plasma treatment to water is shown in Fig. 1b. For irrigation, freshly prepared PAW was always used and all the physiological, molecular and biochemical parameter were assessed in 35 days old seedlings.

Electrical and optical characteristics of plasma instruments. *Electrical characteristics.* The electrical characteristic of the discharge is shown in Fig. 1. The discharge was operated by a controlled circuit with on and off-times of 23.4 ms and 142.0 ms, respectively (Fig. 1c). The duty percentage for this condition is ~14%. A short operational time with long off-times was chosen to operate the discharge for a long time without overheating the electrodes. The current-voltage waveforms appearing during the on-time of the discharge period are shown in Fig. 1d. The voltage waveform appears distorted due to the discharge current peaks appearing in each half cycle of the applied voltage. A positive current peak appears with the increase of the applied voltage during the positive half cycle of the applied voltage. Some charges are accumulated within the dielectric material and are reversed back during the negative half cycle. As a result, a negative discharge current peak appears in the negative half cycle of the applied voltage. Here, the frequency, applied voltage, and current of the discharge are ~83.5 kHz, 0.66 kV, and 70.39 mA, respectively. The dissipated energy (P) per cycle was 3.57 J/s, which was obtained by integrating the current (I(t)) and voltage (V(t)) of one cycle (T) over the complete plasma on time²⁷. Mathematically, P can be expressed as:

$$P = \int_0^T I(t)V(t)dt$$

Optical characteristics. The normalized optical emission spectrum of the discharge for the air soft plasma jet is shown in Fig. 1(e). Weak emission signals from NO γ bands exist in the region between 200 and 280 nm. These

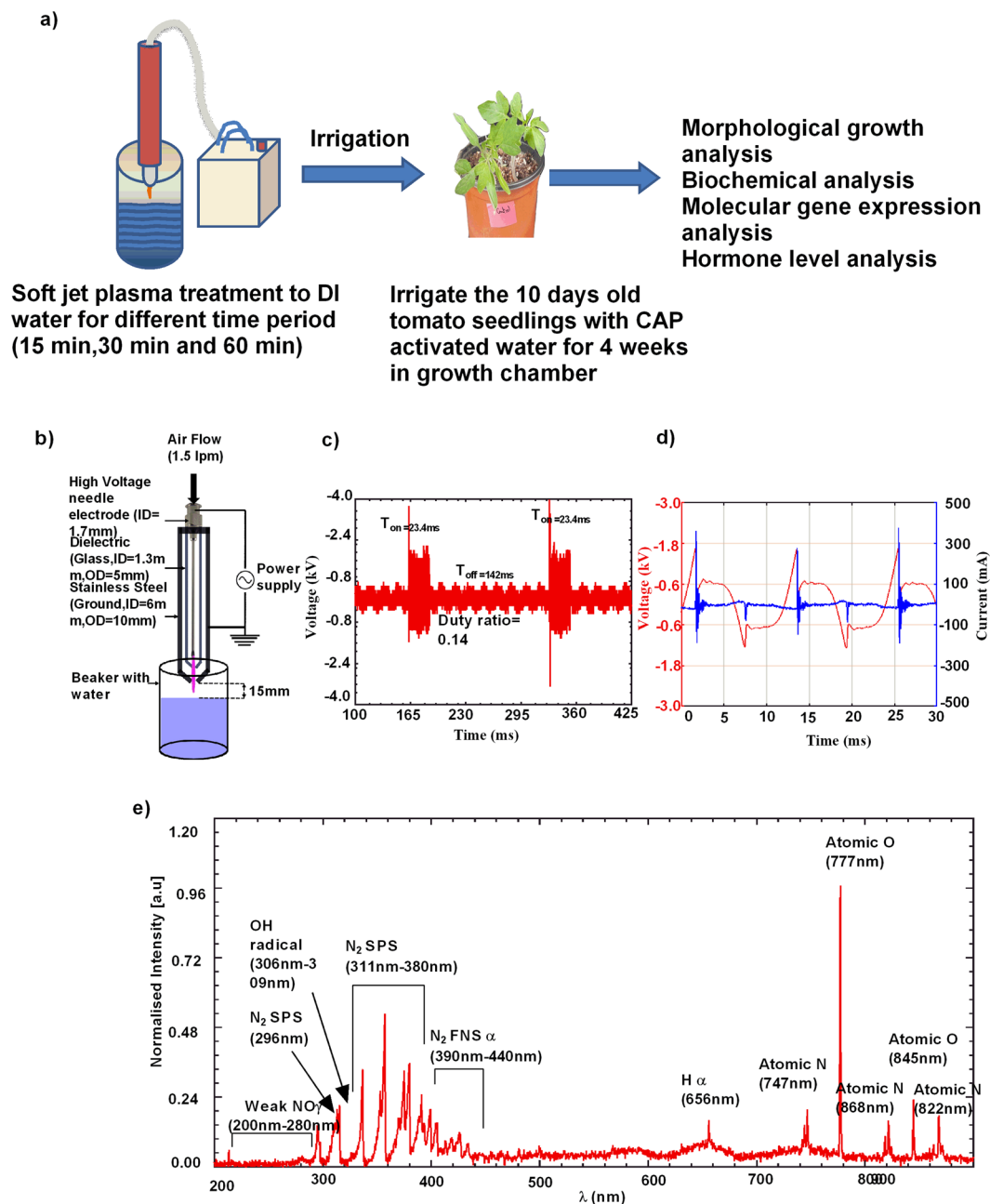


Figure 1. Experimental setup and physical characterization of the jet plasma. (a) Schematic representation of the work plan. (b) Schematic of the experimental setup. (c) Waveforms showing the on and off times of the plasma jet. (d) Current-voltage waveforms of the discharge during plasma on time. (e) Optical emission spectrum of the soft plasma jet with air gas from 200 to 900 nm.

species originate from the collision of energetic electrons with nitrogen and oxygen molecules present in the ambient air²⁸. OH radicals are emitted at approximately 306–309 nm and originated from the collision of electrons, or metastable nitrogen atoms, with water molecules^{27,29}. Strong emissions from various bands of the nitrogen second positive system (N₂ SPS) are observed at 296 nm, 315 nm, 337 nm, 357 nm, and 380 nm.

Furthermore, there are emissions from the nitrogen first negative system (N₂ FNS) between 390 and 440 nm, in addition to atomic nitrogen (742 nm, 822 nm, and 868 nm). The excited nitrogen species originate from the dissociation of nitrogen molecules present in the feeding gas and ambient environment. Strong emission from atomic oxygen (777 nm and 845 nm) is also observed, which is due to the dissociation of oxygen molecules. In addition to these species, emission from the hydrogen alpha line at 656 nm is also observed²⁷.

Chemical properties of water altered by the cold plasma exposure. The interaction of cold air plasma with water activates the production of different RONS in the water, which alters the chemical composition of water²⁹. To determine the effect of CAP exposure on hydrogen ions concentration, the pH of water was

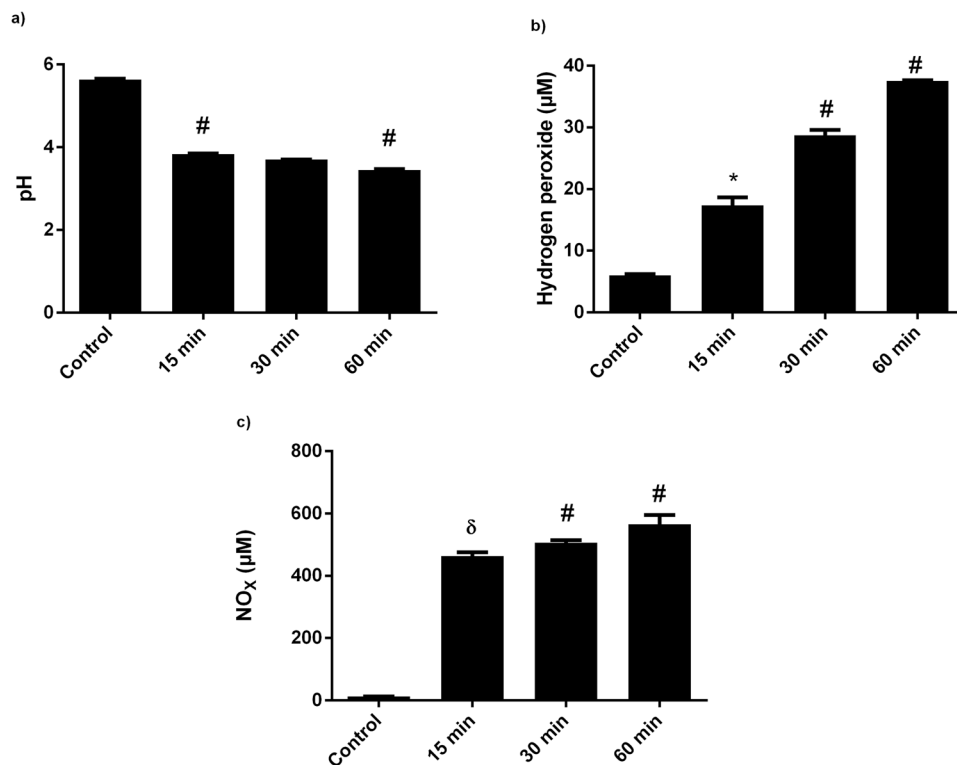


Figure 2. Biochemical properties of PAW. (a) pH, (b) H₂O₂, and (c) NO_x before and after different times of water exposure to plasma. Mean ± SE (n = 3) of each experiment represented in terms of error bars. The SE between the mean of the control and the treatment group was analyzed by student t-test. p-value denoted by * (p < 0.05), δ (p < 0.01), and # (p < 0.001).

measured at different time intervals after CAP exposure. The pH of water after 15 min CAP treatment was 3.8, which, compared to the control (pH of 5.6), is significantly lower. At 30 min and 60 min treatment, the pH of water decreased to 3.7 and 3.45, respectively (Fig. 2a). The CAP-induced changes in the chemical composition of the DI water were estimated by analyzing the H₂O₂ and NO amounts using a spectrophotometer. Compared to the control, the concentration of H₂O₂ increased significantly in all PAWs. The CAP exposure of 15 min, 30 min and 60 min to deionized water increased the H₂O₂ concentration 1.8-, 3.7-, and 5.25-fold, respectively (Fig. 2b). Similarly, the NO_x concentration also increased with increasing CAP exposure time. The NO_x concentration increased 35, 38, and 43-fold, compared to the control after the CAP treatment in water for 15 min, 30 min, and 60 min, respectively (Fig. 2c). The CAP produced various RONS in the gaseous phase, which, on interaction with water, produced long-lived RONS species and lowered the pH^{30–32}.

PAW irrigation induces growth of tomato seedlings. The growth-inducing effect of PAW in seedlings was examined both morphologically and phenotypically. The tomato seedlings irrigated with 15PAW and 30PAW showed better morphological growth compared to the control seedlings (Fig. 3a). The phenotypically seedling growth was measured in terms of shoot and root lengths (Fig. 3b). Compared to the control samples, 15PAW and 30PAW-treated seedlings showed greater shoot and root length, whereas a non-significant change was observed in the 60PAW. The ratio of the shoot to root length maximum after the 30PAW treatments (Fig. 3c). These results indicate that PAW treatments enhance the growth of seedlings. Similar observations of the effect of PAW irrigation on lentil seedlings length was published by^{33,34}. Previous publication³³ reported that PAW-treated lentil seedlings exhibited 34% and 128% increase in the seedling length after the 3rd and 6th days of irrigation. Few reports also observed that the air PAW induced significant seedling growth compared to O₂, N₂, and He PAW³⁵.

Enhancement of endogenous RONS species by PAW. To determine the effect of PAW treatments on the plant's endogenous RONS concentration, H₂O₂ and NO_x were directly chemically detected in leaves and roots (Fig. 4). *In vivo*, H₂O₂ reacts with DAB and produces brown precipitates whose intensities were measured by ImageJ software and were used to prepare a graph. *In vivo* examination of H₂O₂ in leaves showed that, compared to the control samples, the H₂O₂ stain area increases in all PAW-treated plants (Fig. 4a). The spot intensity of 15PAW, 30PAW, and 60PAW seedlings leaves significantly increased compared to the control. Similarly, the PAW-treated roots had a higher H₂O₂ stain intensity compared to non-PAW-treated plants (Fig. 4b).

In vivo RNS content was detected in leaves and roots of seedlings using a fluorescent dye 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM Diacetate) and fluorescent images were captured by confocal microscopy. Corrected total cell fluorescence (CTCF) of RNS in leaves and roots was measured by ImageJ software. The CTCF of RNS increased significantly in the root of all PAW-treated tomato seedlings

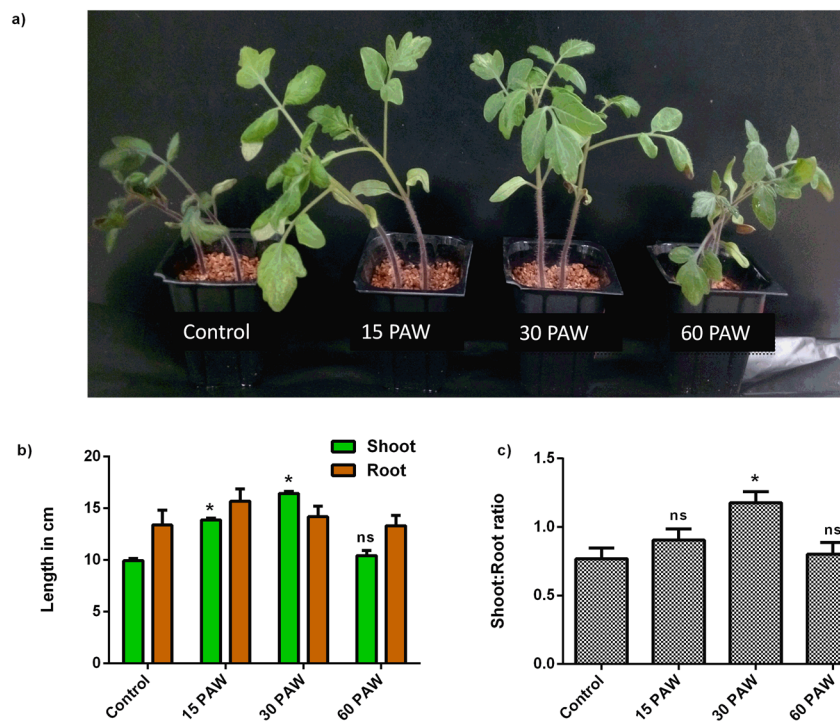


Figure 3. Effect of PAW treatment at different time-intervals on (a) physiological status of tomato seedlings (28 days old) grown in vermiculite. (b) shoot and root length (cm) and (c) shoot: root length ratio. \pm SE of mean ($n = 3$) of each experiment represented in terms of error bar. Significant difference between the mean of control and treatment group was analyzed by student t-test. p-value denoted by * ($p < 0.05$), δ ($p < 0.01$), and # ($p < 0.001$).

(Fig. 4c), whilst in leaves, a non-significant induction in CTCF values of RNS was observed in 15PAW and 30PAW seedlings. A significant alleviation in CTCF of RNS was observed in the 60PAW plants (Fig. 4d).

H_2O_2 plays a very crucial role in the cascade of the free-radical reaction mechanism. Hence, it was assessed in the shoot and roots of tomato seedlings. H_2O_2 concentration increased in 15PAW, 30PAW, and 60PAW seedlings. H_2O_2 levels in 15PAW, 30PAW, and 60PAW shoot increased 0.14-, 0.22-, and 0.32-fold, respectively, compared to the control (Fig. 4e). In seedling roots, 15PAW and 60PAW exhibited similar patterns of H_2O_2 induction (0.19 fold and 0.23 fold), whereas 30PAW roots had no significant difference in H_2O_2 concentration compared to control seedlings (Fig. 4e).

The RONS species generated in water by plasma treatment also enhanced the endogenous level of NO_x in plants. In 30PAW and 60PAW seedlings, the shoot showed a significant improvement (10.7 fold and 18.3 fold) in NO_x concentration compared to the control, whereas the 15PAW showed a smaller increase in NO_x concentration. Similar upregulation of NO_x concentration was observed in PAW-treated tomato seedlings roots. In 30PAW and 60PAW seedlings roots, the NO_x level increased up to 1.9 and 4.4-fold, respectively, compared to the control (Fig. 4f). These results suggest that PAW-induced RONS positively influence the endogenous RONS status of seedlings.

15 min and 30 min PAW irrigation induce RONS signaling, not RONS toxicity, in tomato seedlings.

Several biochemical markers of stress were analyzed in shoots and roots of seedlings to measure the oxidative damage potential of PAW-induced RONS in the plant cells. Proline, an imino acid, acts as an osmolyte and redox buffer in cytoplasm³⁶. Proline content of 15PAW and 30PAW shoots increased significantly but reduced in 60PAW shoots compared to control. In roots, no significant changes were observed. Compared to the control root, the proline content of 15PAW and 30PAW seedlings roots was slightly lower, but it was 0.5-fold higher in the 60PAW seedlings (Fig. 5a). Lipid Peroxidation is an indicator of oxidation of lipids membranes by RONS and, in this experiment, was detected by analyzing the accumulation of MDA, a byproduct of lipid oxidation reaction in plants. The MDA content of the 15PAW and 30PAW shoots was lower (0.44-fold and 0.64-fold) than in the control shoot. However, the MDA content of the 60PAW shoot increased 0.39-fold, compared to the control. In roots, the MDA content was lower in the 15PAW but was not significant in the 30PAW and 60PAW with respect to the control roots (Fig. 5b). Ascorbic acid levels were also determined in PAW-treated plant spectrophotometrically. 15PAW, 30PAW, and 60PAW leaves increased the ascorbic acid content by 0.07, 0.48, and 1.15-fold, respectively. However, a reduction in the ascorbate content was observed in the roots of 15PAW and 30PAW (Fig. 5c). The chlorophyll content is a measure of photosynthetic activity and its presence was enhanced in the 30PAW, while it was reduced in the 60PAW, compared to the control (Fig. 5d).

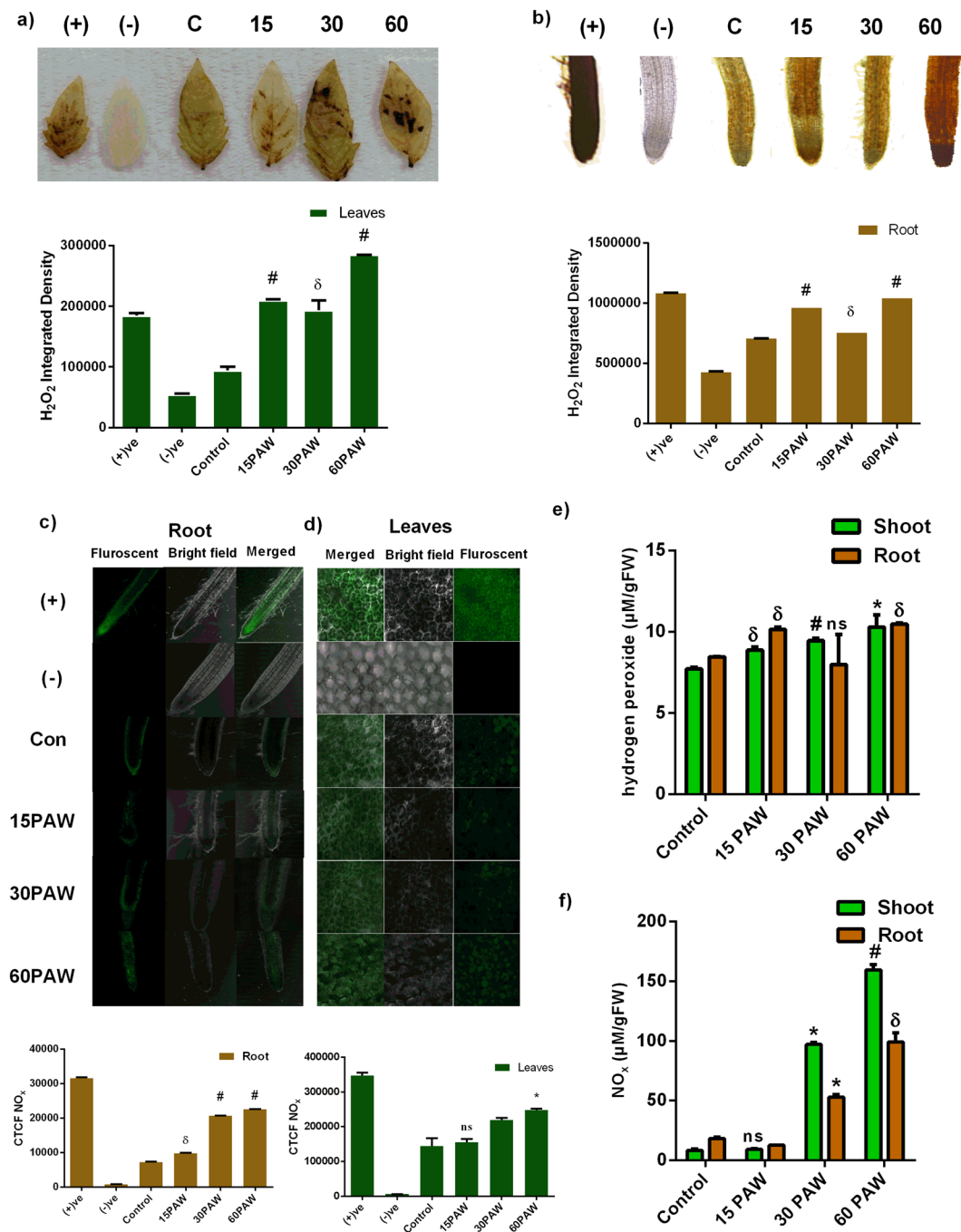


Figure 4. Biochemical representation of endogenous H₂O₂ in (a) leaves and (b) root tips. Microscopic NO_x detection in tomato seedlings (c), leaves, and (d) root tips. Spectrophotometric quantitation of (e) H₂O₂ and (f) NO (NO_x) in seedlings. \pm SE of mean (n = 3) of each experiment represented in terms of error bar. The significant difference between the mean of the control and the Paw-treated group was analyzed by student t-test. The p-value denoted by * (p < 0.05), ^δ (p < 0.01), and [#] (p < 0.001).

These results indicate that the 15PAW and 30PAW treatments do not induce any oxidative damage and the 30PAW irrigation improved the antioxidant potential and reduced oxidative toxicity. Longer exposures (60 min) of CAP to water produced excessive RONS in 60PAW, which initiated the RONS stress or toxicity. This suggests that shorter exposure of water to CAP (15 min and 30 min) produces amounts of RONS that activate plant growth but are not toxic.

PAW up-regulated defense response and antioxidant gene expression. To understand the effect of the PAW treatment on plant defense and antioxidant potential, the expression of various genes related to plant redox homeostasis and pathogenesis resistance were examined by qPCR. Pathogenesis related gene expression

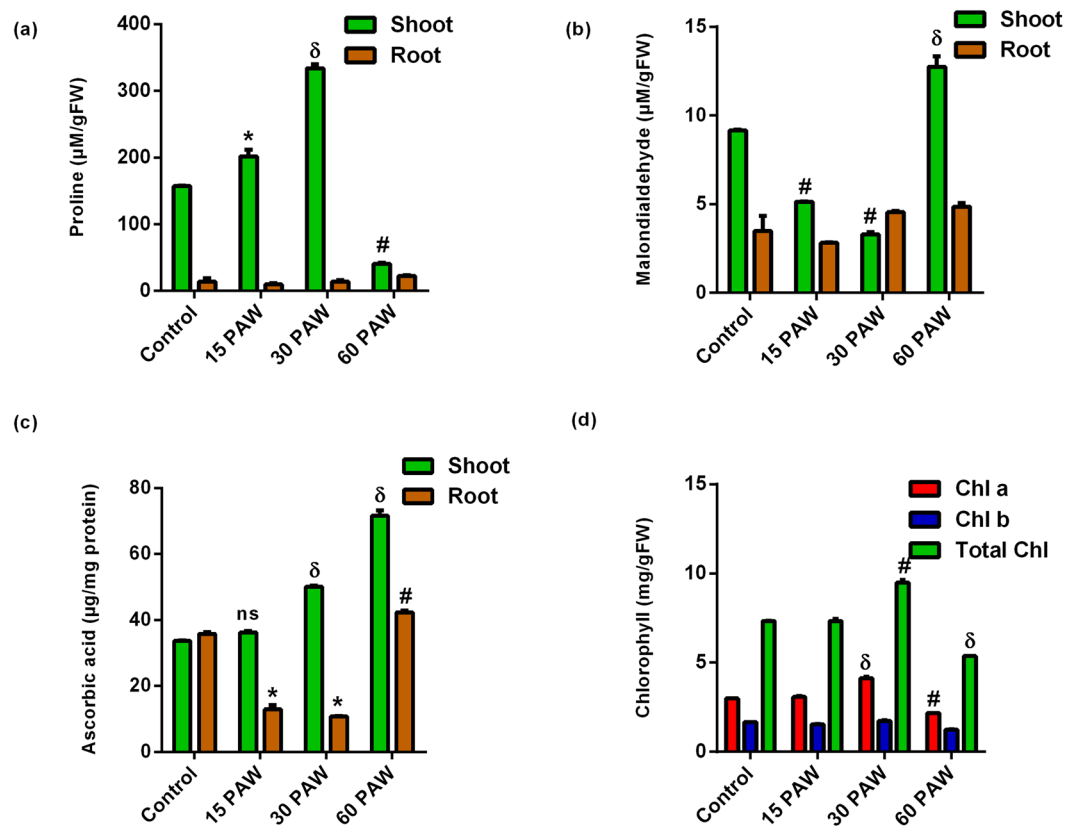


Figure 5. Biochemical status of tomato seedlings treated with 15PAW, 30PAW, and 60PAW. Content of (a) Proline, (b) Malondialdehyde (c) Ascorbic acid, and (d) chlorophyll in shoot and roots. \pm SE of mean ($n = 3$) of each experiment represented in terms of error bar. The significant difference between the mean of the control and the treated group was analyzed by student t-test. p-value denoted by * ($p < 0.05$), δ ($p < 0.01$), and # ($p < 0.001$).

elicits in response to pathogen invasion in plant cells via MAPK signaling³⁷. Chitinase 3 acidic and β -1, 3 glucanase is antifungal proteins that hydrolyze the cell wall of a pathogen. β -1, 3 glucanase is also involved in molecular trafficking through plasmodesmata and flower formation^{38,39}. β -1, 3 glucanase gene expression was lower in 15PAW and 30PAW than in 60PAW leaves (Fig. 6a). However, 15PAW and 30PAW roots showed higher expression of β -1, 3 glucanase gene expression (Fig. 6d). Expression of chitinase 3 acidic gene significantly upregulated in leaves of 30PAW and 60PAW (Fig. 6b) but in the roots of PAW treated seedlings, its expression was reduced successively in 15PAW, 30PAW, and 60PAW compared to non-PAW treated seedlings (Fig. 6e). The MAPK responsible to transduce the signaling by phosphorylating the other signaling pathway proteins showed higher expression at 30PAW, while its expression level decreased in 60PAW seedlings leaves (Fig. 6c). The *mapk* gene expression in roots showed similar patterns to the leaves in which 30PAW showed a higher gene expression than the 15PAW treated plant roots, which again declined at 60PAW (Fig. 6f).

To maintain the redox homeostasis in plant cells, antioxidant enzymes are expressed continuously. The expression of antioxidant enzymes enhances as the level of RONS content is induced in biotic stress conditions⁴⁰. SOD enzymes detoxify the superoxide radical and produce H_2O_2 . The expression of the *sod* gene upregulated one fold at 30PAW leaves and 2.4-fold at 30PAW root, compared to the control seedlings. The SOD expression was lower in 15PAW leaves but induced a 0.5-fold increase in 15PAW roots. In 60PAW leaves, the *sod* gene expression of seedlings decreased significantly compared to the 30PAW and was comparable to the control, while in roots it was comparable to the 15PAW (Fig. 6g,j). The Catalase enzyme catalyzes the quenching of H_2O_2 during photorespiration and is responsible for H_2O_2 decomposition in plant cellular organelles, such as peroxisome and mitochondria⁴¹. Catalase expression was the highest in 30PAW seedlings and it was 6.5-fold in leaves and 1.4-fold in roots, compared to the control (Fig. 6h,k).

In the 60PAW seedlings, *cat* expression increased 3.9-fold in leaves but decreased significantly in roots compared to the control. Glutathione S transferase also quenches RONS and protects the plants from oxidative stress. Glutathione S transferase (*gst*) expression was upregulated in 30PAW leaves (0.9-fold) but decreased in seedlings leaves treated with 60PAW. In 15PAW roots and 30PAW seedlings, gene expression induced a 3.7-fold and 4-fold, respectively (Fig. 6l,i).

PAW treatment enhances SA and JA content in tomato seedlings. Phytohormones regulate various developmental, metabolic, and defense mechanisms of plants⁴². SA and JA are phytohormones synthesized

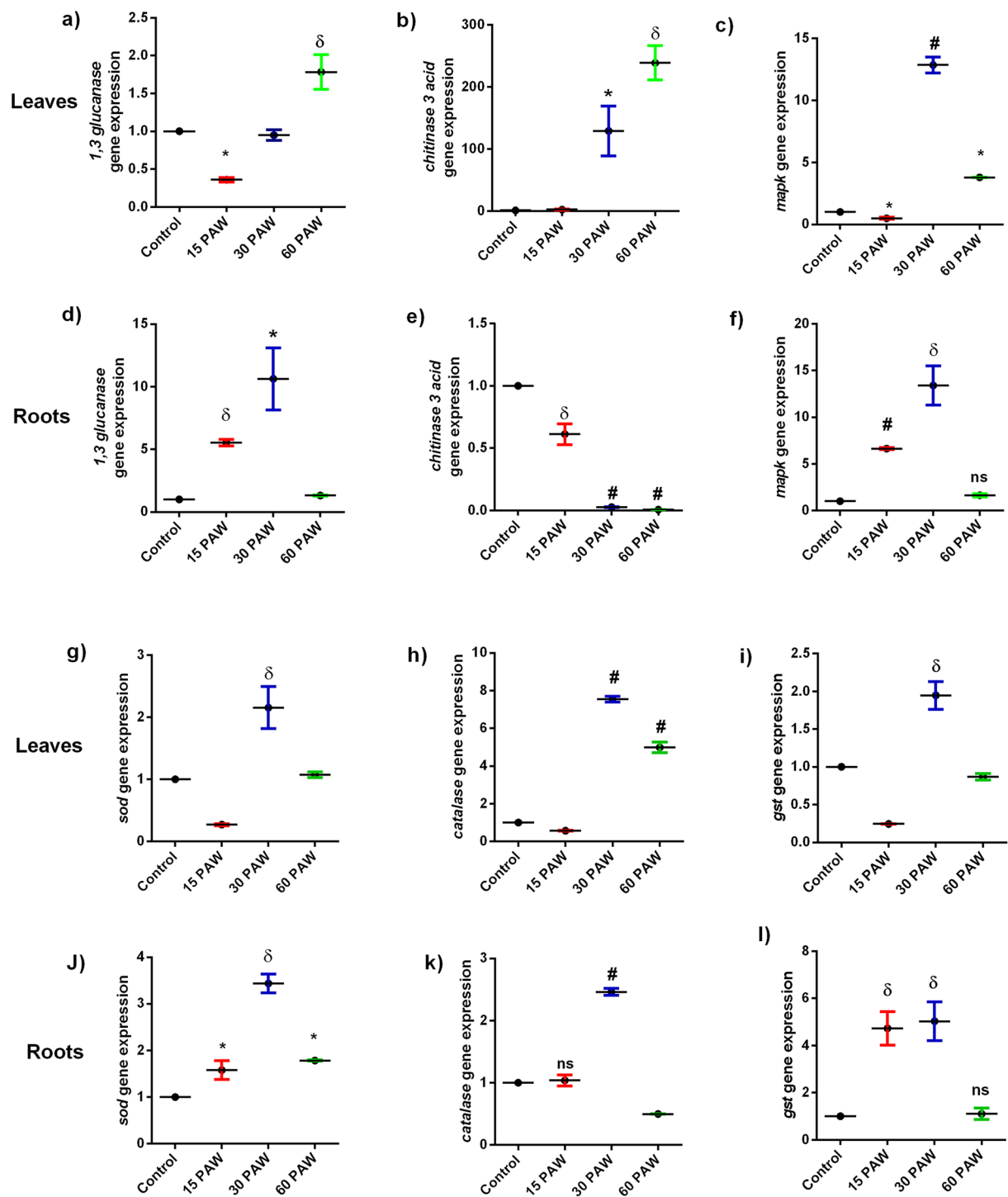


Figure 6. Quantitative PCR for gene expression analysis of different pathogenesis gene. β -1-3 glucanase (a): leaves, (d): roots), Chitinase 3 acid (b): leaves, (e): roots), MAPK (c): leaves, (f): roots), and redox homeostasis genes, such as Superoxide dismutase (g): leaves, (j): roots), Catalase (h): leaves, (k): roots), Glutathione S-transferase (i): leaves, (l): roots) in different PAW treatments in tomato seedlings. \pm SE of mean ($n = 3$) of each experiment represented in terms of error bar. The significant difference between the mean of the control and the treated group was analyzed by student t-test. p-value denoted by * ($p < 0.05$), δ ($p < 0.01$), and # ($p < 0.001$).

in response to biotic stress, such as pathogens and herbivores attacks. 12-Oxophytodienoic acid reductase (*opr1*) and allene oxidase synthase (*aos*) are regulatory genes of JA synthesis⁴³. The mRNA level of *opr1* and *aos* were induced in leaves by the PAW treatment. The expression of *opr1* gene induces in 15PAW, 30PAW, and 60PAW leaves compared to the control (Fig. 7a). In the roots of the PAW treated seedlings, gene expression significantly

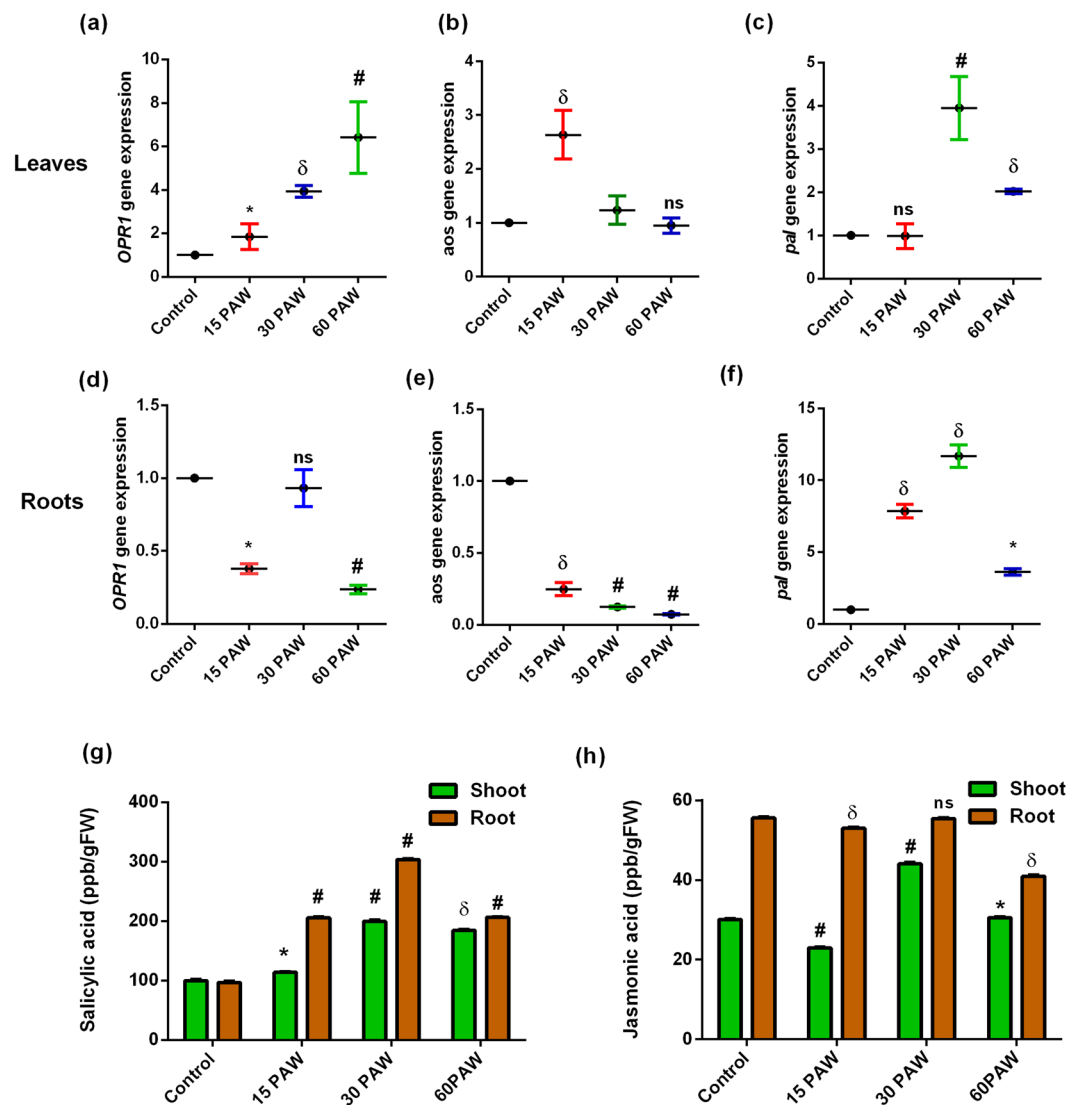


Figure 7. SA and JA status in PAW treated tomato seedlings. Gene expression analysis of key enzymes of JA and SA synthesis pathways. 12-Oxophytodienoic acid reductase (*opr 1*) (a): leaves, d: roots), Allene oxidase synthase (*aos*) (b): leaves, (e): roots) and Phenylalanine ammonia lyase (*pal*) (c): leaves, f: roots). LC-MS analysis of SA and JA content. (g) SA content in leaves and root and (d) JA content in leaves and roots of tomato seedlings. \pm SE of mean ($n = 3$) of each experiment represented in terms of error bar. The significant difference between the mean of the control and the treatment group was analyzed by student t-test. p-value denoted by * ($p < 0.05$), δ ($p < 0.01$), and $\#$ ($p < 0.001$).

reduced as a result of the 15PAW and 30PAW treatments with respect to control (Fig. 7d). Allene oxidase synthase (*aos*) gene expression level was the highest in the 15PAW leaves and was reduced in the roots of all PAW treatments (Fig. 7b,e). In the 30PAW and 60PAW leaves, no significant difference in *aos* mRNA profiling was detected. Phenylalanine ammonia Lyase (PAL) catalyzes the first step of SA and other phenolic compound synthesis⁴⁴. Phenylalanine ammonia lyase expression was maximum in the 30PAW (2.9-fold) followed by the 60PAW leaves (1.0-fold) (Fig. 7c). Similarly, the highest *pal* gene expression (10.6-fold) was observed in the 30PAW followed by the 15PAW and 60PAW roots (6.8-fold and 2.6-fold, respectively) compared to control seedlings roots (Fig. 7f).

LC-MS is a fast and sensitive method to detect the phytohormone content in plant extracts⁴⁵. Therefore, LC-MS of PAW treated seedlings (leaves and roots) was performed to validate the qPCR results. A standard graph for both hormones, SA and JA, was prepared using various concentrations (5 ppb, 10 ppb, 20 ppb, 50 ppb, 100 ppb, 500 ppb, and 1000 ppb) of the standard solution. SA showed the peak at retention time: 6.36 min and JA peak confirmed at RT: 7.42 min in the chromatogram (Supplemental figure S2). The SA content was upregulated in all PAW treatments and was maximum in 30PAW seedlings leaves (0.99-fold) and roots (2.14-fold) (Fig. 7g). JA followed a similar pattern of SA in the PAW treated seedlings leaves. In the 30PAW leaves, the JA content induced the highest (0.44-fold) as compared to control. On the other hand, no significant effect on the JA content of the 15PAW and 30PAW seedling roots was observed, while in the 60PAW the JA concentration was lower than in the control plant (Fig. 7h). AOS and OPR1 are two key enzymes of the JA biosynthesis pathway. Both of these

enzymes expression affects the JA biosynthesis. In the present study, 30PAW irrigation induced expression of AOS and OPR1 gene in leaves which mutually leads to the induction in JA. Although, 15PAW and 60PAW showed only significant induction either in AOS or OPR1 gene hence less regulating effect on JA biosynthesis and content. Similarly in the case of roots, lower expression of AOS and OPR1 enzyme which leads to a significant and non-significant reduction in JA content.

All these results indicate that the irrigation of tomato seedlings with PAW leads to many physiological, biochemical, molecular, and hormonal alterations. The 15PAW treatment showed a positive stimulatory effect on plant growth. The 30PAW treated seedlings showed better growth, antioxidant, hormone status, and defensive gene expression than all other PAW-irrigated and control seedlings. The 60PAW treated seedlings had higher antioxidant and PR gene expression with the accumulation of higher RONS, which resulted in more lipid peroxidation and chlorophyll degradation.

Discussions

The present study was designed to investigate the effect of PAW irrigation on the plant defense system. This study also links the RONS generated by CAP treatment to the modulation in plant defense system. The choice of feeder gas plays a vital role in generating the diversity of RONS in CAP. It is already documented that the use of air as feeder gas in CAP produces a greater variety of RONS than other gases, such as argon, N₂, and O₂³⁵. Here, the optical emission spectra of air jet CAP revealed the presence of various peaks of hydroxyl radicals, nitric oxide, atomic oxygen, atomic nitrogen, and reactive nitrogen molecule species. These species interact with water molecules and contribute to the alteration of the physicochemical properties of water. Interaction of water with these CAP generated reactive hydroxyl radicals, atomic oxygen, nitrogen, and active molecule nitrogen species leads to the production of other RONS, such as H₂O₂ and NO_x. H₂O₂, NO, nitrate ions, and peroxyxynitrous acid are predominantly produced within PAW and contribute to acidifying water. Although in the present study we focus mainly on the presence of H₂O₂ and NO_x in PAW, other species were observed in PAW by³³.

These RONS contribute to both reducing the pH of the PAW and modifying its chemical composition, hence affecting the plant growth. Sivachandiran⁴⁶ also reported a similar decrease in pH of water on plasma treatment and its seedling growth-inducing effect in radish (*Raphanus sativus*), tomato (*Solanum lycopersicum*), and sweet pepper (*Capsicum annuum*). The induced concentration of NO_x in PAW was expected to have a growth-inducing impact on the seedlings. Previous reports also suggest that nitrite and nitrate ions in PAW act as nitrogen fertilizers and contribute to the enhancement of seedlings growth⁴⁷. OES spectrum of air jet plasma showed the presence of atomic oxygen which can contribute in the generation of molecular oxygen in PAW. Molecular oxygen is a well-known product of chemical reactions occurring in PAW³⁰. Papadopoulos⁴⁸ reported that molecular oxygen improved the nutrition absorption efficiency of roots. The present investigation suggested that another possible explanation of this growth enhancement is the higher oxygen content of PAW, which improves the nutrition uptake capacity of roots. Thus, both nitrate ions and molecular oxygen are required to induce the seedling growth.

In the present study, PAW irrigation enhanced endogenous H₂O₂ and NO_x levels in seedlings. Hence, RONS present in plasma treated water influence the endogenous RONS level. These observations were supported by Mejia-Teniente, 2013⁴⁹ study, where the exogenous application of 14 mM and 18 mM H₂O₂ solution induced the endogenous H₂O₂ content of *Capsicum annuum* plant. The exposure time of the plasma treatment of water plays an important role in regulating the concentration of RONS in water and plants. Shorter exposure to plasma, such as 15 min and 30 min, produced sufficient RONS to induce RONS signaling in seedlings. At the basal level, H₂O₂ and NO RONS act as signaling molecules in plants². Longer exposure to plasma (60 min) generates excessive RONS and initiates the oxidative stress in the seedlings. Hence, 15PAW and 30PAW seedlings have a better biochemical stress and growth profile than the control and the 60PAW. The RONS in PAW-treated seedlings initiate RONS signaling and gene expression, and lead other biochemical changes in the plant cell. In previous studies, the pretreatment of H₂O₂ solution to soybeans seedling enhanced the endogenous H₂O₂ content and increased the chlorophyll content and lipid peroxidation of soybean leaves⁵⁰. Jiang⁵¹ reported higher accumulation of H₂O₂ and antioxidants (peroxidase and polyphenol oxidase) activity in plasma treated tomato seedlings. Likewise^{34,52} reported that seeds exposure to cold plasma exposure induce proline, total soluble sugar content, antioxidant genes, PR genes, and reduce the MDA content in seedlings. Therefore, it is proposed that H₂O₂ and NO_x present in PAW and exogenously applied H₂O₂ and NO_x solution may follow similar mechanisms of action and have a similar impact on plant cells.

Transcription regulation studies of H₂O₂ and NO in tobacco plants revealed that H₂O₂ and NO mutually regulate expression of defense related genes^{49,53}. In the present investigation, we observed that mitogen activated protein kinase (*mapk*) gene expression was induced in both root and shoot of PAW treated tomato seedling. Capone^{50,54} found that MAPK induction in the shoot in response to H₂O₂ and NO was similar in both conditions when applied through root or direct injection to leaves and proposed that the transmission of exogenous H₂O₂ and NO signals from root and shoots. From the present study, as well as previous reports, it is clear that H₂O₂ and NO both induce the MAPK signaling pathway and regulate the expression of defense related genes. Higher expression of defense related gene expression was observed in leaves of PAW treated seedlings³⁵. Here, a diverse profile of biochemical markers and gene expression was observed in roots and leaves of PAW-treated seedlings. Earlier studies on plant immune response reported similar tissue specific differential immune responses of roots and suggested the compartmentalization of defense mechanisms in root systems⁵⁶. Panngom⁵⁷, observed that direct treatment of cold plasma to tomato leaves induces the gene expression in the roots only. Therefore, in light of the present observations and earlier reports we propose that RONS transmit from the site of exposure to other parts of the plant and are differentially regulated at the molecular and biochemical levels.

Plants synthesize various phytohormones in response to biotic stress which upregulates the production of small defense proteins and strengthens the plant's immune system. SA and JA are key regulatory immune phytohormones¹³. When a pathogen invades the host system, it firstly induces endogenous RONS levels, which trigger

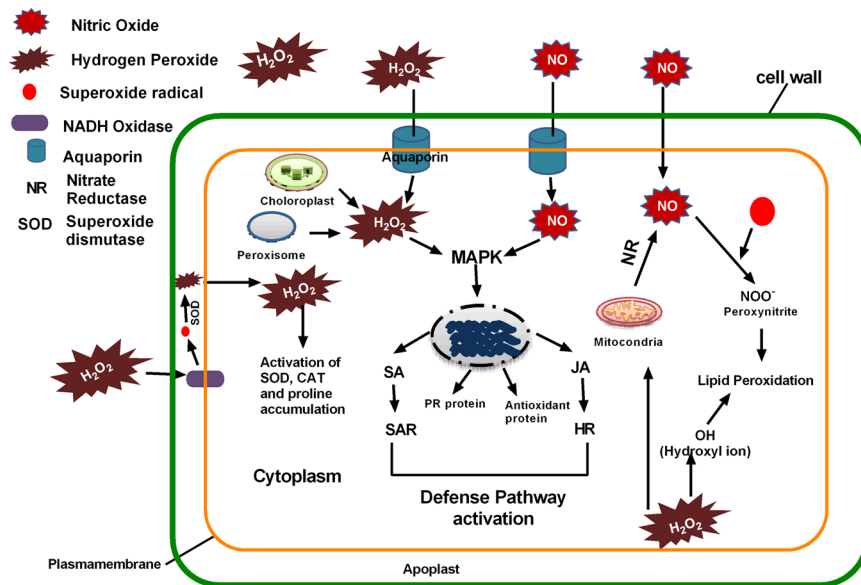


Figure 8. Schematic representation of PAW generated RONS and RNS effects on plant cellular metabolism. PAW H₂O₂ and NO either transport via aquaporin or induce the production of RONS in cytoplasm via NADH oxidase. H₂O₂ and NO initiate the production of other RONS in cytoplasm and enhance intracellular RONS concentration. These RONS activate defense pathway genes by inducing PR gene expression, antioxidant gene expression, SA, and JA content.

hormone synthesis regulatory genes expression, defensive protein, and PR genes synthesis. These synthesized genes kill the pathogen and prevent it from infecting other cells by hypersensitive responses and SAR⁷. In the present investigation, PAW irrigation stimulated SA and JA synthesis pathway enzymes expression and increased their content in plant leaves and roots. H₂O₂ and NO are the primary reactive species generated in seedlings by PAW irrigation. H₂O₂ triggered the expression of SA synthesis pathway genes and its accumulation in cell⁵⁸. SA induced PR gene expression and led to SAR. NO induced the expression of pathogenesis related proteins, antioxidant enzymes, root growth, and JA synthesis⁵⁹. In *Arabidopsis thaliana*, JA synthesis pathway genes expression was upregulated in response to NO content⁹. Here, we proposed that PAW-mediated H₂O₂ and NO_x induction in plants mimics the RONS burst via pathogen attack and exhibit changes in gene expression.

In summary, all the biochemical events and modifications by RONS (H₂O₂ and NO) in plant cells are demonstrated in Fig. 8. Cold plasma treated-water irrigation induced H₂O₂ and NO levels increase in the extracellular space. From the extracellular space H₂O₂ and NO either directly transport into cell cytoplasm through aquaporin or induce the production of H₂O₂ from NADH oxidase, chloroplasts, peroxisomes, and NO from mitochondria. H₂O₂ and NO both activate the production of other RONS, such as superoxide ion, peroxynitrite, and hydroxyl radicals in the cytoplasm, which causes lipid oxidation at higher concentrations. H₂O₂ and NO both induce the expression of PR proteins, antioxidant enzyme activity, SA and JA pathway enzymes via MAPK signaling pathways. All these defense proteins and phytohormones strengthen the plant pathogen defense pathway.

Overall, the present study suggests that PAW can act as both plant growth inducer as well as immune inducer. Therefore, in the future, studies on cold plasma generated RONS signaling in plants need to be emphasized for a better understanding of the potential of cold plasma in plant growth, development, and defense.

Materials and Methods

Characterization of the CAP device and water treatment. A schematic of the experimental setup is shown in Fig. 1(a). The plasma jet consists of a high voltage needle electrode (ID = 1.7 mm), which is inserted inside a glass dielectric tube (ID = 3.3 mm, OD = 5 mm). The glass tube is inserted inside a cylindrical stainless-steel metal electrode (ID = 6 mm and OD = 10 mm), which is connected to the ground. The distance from the end of the dielectric glass to the end of the grounded metal electrode is 1 mm. Air gas at a flow rate of 1.5 liters per minute is used through the inner needle electrode as a feeding gas. Discharge occurs when the alternating voltage is applied between the power needle electrode and the outer grounded electrode. To produce the PAW, a 50-ml beaker is filled with de-ionized (DI) water and the distance from the end of the grounded electrode to the top of the water surface is kept at 15 mm (Fig. 1a) and treated at the following exposure times: 15 min, 30 min, and 60 min.

Assessment of plant growth. Tomato (*Solanum lycopersicum*) was used to investigate the effect of PAW on the phytohormone status. Tomato (cultivar named “titichal”) seeds were obtained from Nongwoo Bio, Suwon, South Korea. They were first germinated in petriplates on wet paper towels and left in darkness at room temperature for two days. The germinated tomato seeds were then transferred into a vermiculite pot and grown in a growth chamber under controlled conditions (temp: 22 °C, humidity: 60%, photoperiod: 16/8 hours light/dark).

After ten days of germination, the tomato seedlings were irrigated with 10 ml of PAW, whilst control seedlings were irrigated with DI water. Plasma-activated water treatments were performed two times per week for up to five weeks. Subsequently, after the completion of the treatment, the five weeks old (35 days) tomato seedlings were used for physiological, biochemical, molecular, and phytohormone analysis (Supplemental figure S1). Seedlings irrigated with PAW with 15 min, 30 min and 60 min treated plasma activated water are referred to as 15PAW, 30PAW, and 60PAW, respectively.

Estimation of H₂O₂ and NO_x. Endogenous H₂O₂ of tomato seedlings and PAW were detected spectrophotometrically using QuantiChrom™ Peroxide assay kit (Bioassay Systems, USA). In this assay, a purple color complex was formed after oxidation of Fe²⁺ into Fe³⁺ by H₂O₂ (Fe³⁺-xylenol orange reaction), which was measured at 540–610 nm. *In vivo* visualization of H₂O₂ in tomato leaves and roots was carried out using 3,3-diaminobenzidine (DAB) as substrate⁶⁰. Firstly, leaves and roots were carefully excised from seedlings and immersed in DAB solution (1 mg/ml) under darkness for eight hours. DAB reacted with H₂O₂, resulting in a brown-colored product in seedlings leaves and roots. Next, for better visualization of the brown spots, chlorophyll was removed by immersing leaves in ethanol (96%) and keeping them at 65 °C in an incubator for one hour. Finally, leaves and roots were washed twice with ethanol and were photographed. Prior to DAB staining, positive control leaves and roots were incubated with 10 mM H₂O₂ for 10 min. Microscopic images were analyzed by ImageJ software (<https://imagej.nih.gov>) to determine the respective DAB staining intensities.

The NO_x amount was measured in plants and PAW by QuantiChrom™ NO assay kit (Bioassay Systems, USA)⁶¹. The NO level was measured by the reduction of nitrate to nitrite using the Griess method at 540 nm spectrophotometry. Endogenous detection of reactive nitrogen species RNS in seedlings roots and leaflets were performed by the DAF-FM Diacetate (4-Amino-5-methylamino-2,7-Difluorofluorescein Diacetate) (ThermoFisher Scientific, USA) fluorescence dye using an Olympus IX83-FP confocal microscope (Olympus, Japan)⁶². For endogenous detection of NO, plant roots and leaves were excised and washed with DI water. Five-mm root tips were cut and the leaf abaxial epidermis was peeled before immersing it in 10 μM of DAF-2DA (prepared in 10 mM MES-KCl buffer, pH 7) followed by 5 min incubation in a vacuum chamber and 15 min at room temperature in a dark chamber. After incubation, plant samples were thoroughly washed with 10 mM MES-KCl buffer and examined by epi-fluorescence using alexa fluor 488 (excitation 495 nm and emission 515 nm). Positive control leaf and root samples were treated with 100 μM sodium nitroprusside and negative control samples were pre-incubated with 100 μM cPTIO for 2 hours. Corrected total cell fluorescence (CTCF) analysis of the microscopic images was performed using ImageJ software.

Biochemical assays. *Malondialdehyde (MDA) content.* MDA content in plants is a direct measure of lipid peroxidation by RONS. MDA content was determined as in⁶³. Tomato seedling (leaves and roots) samples (0.5 gm) were powdered using liquid N₂ and were homogenized with 4 ml of 20% Trichloroacetic acid containing 0.5% Thiobarbituric acid. The homogenized samples were incubated at 95 °C in a water bath for 15 min. Samples were immediately transferred to an ice bath for cooling and centrifuged at 10,000 × g for 10 min. The absorbance of 1 ml supernatant was measured by a spectrophotometer at 532 nm.

Proline content. Free proline content in plants was measured by the procedure reported earlier⁶³. Leaf and root samples (0.5 gm) were ground into a fine powder using liquid N₂ and were mixed with 2 ml of 3% sulphosalicylic acid in a tube. Homogenized samples were centrifuged at 10,000 × g for 5 min. Acetic acid and ninhydrin reagent (500 μl each) were mixed with supernatant, placed in a boiling water bath for 45 min, and then immediately transferred in ice. An equal amount of toluene was added to each sample, which was then vortexed. The optical density of the upper layer (toluene) was measured at 520 nm by a spectrophotometer.

Chlorophyll content. The chlorophyll content was measured by DMSO protocol⁶³. Tomato leaves (75 mg) were cut into uniform disc-shaped samples and submerged in 10 ml of dimethylsulphoxide. The tubes were incubated at 65 °C for 4 h. The concentration of chlorophyll a, chlorophyll b, and total chlorophyll were calculated by measuring the absorbance at 663 and 645 nm.

Ascorbate content. Ascorbic acid content in tomato leaves and roots was measured colorimetrically using a vitamin C assay Kit (Elabscience, USA). This assay kit is based on the reduction of Fe³⁺ into Fe²⁺ by ascorbic acid and the color developing reaction of Fe²⁺ with phenanthroline. The amount of color production is directly proportional to the amount of ascorbic acid in plants and was measured at 536 nm.

qPCR for measuring gene expression. Gene expression studies of PR genes, antioxidant enzymes, and hormone synthesis pathway enzymes (Supplemental Table 1) were executed through quantitative PCR (qPCR). The total RNA was isolated from the shoots and roots of tomato seedlings using RNAiso Plus (TAKARA BIO INC, Japan) and converted into cDNA using oligo dT primer by ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover kit (Toyobo Co. Ltd., Japan). For qPCR, 20-μl reaction mixtures were prepared including 2x SYBR Green master mix (iQ™ SYBR[®] Green supermix BioRad), 0.3 μM of each primer, 1 μl of cDNA, and DNase/RNase free water. The qPCR was performed in 96 well plates for 40 cycles at PCR cycle conditions (95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s) by CFX96™ Real-Time System (BioRad) thermocycler. The 18S rRNA gene was used as an endogenous control. All the sample reactions were performed in triplicates two times.

Liquid chromatography-mass spectrometry analysis of SA and JA. Endogenous plant hormone levels in both treated and control tomato seedlings were analyzed by liquid chromatography-mass spectrometry (LC-MS). The plant samples extracted for hormone LC-MS analysis were prepared by manual protocol⁶⁴.

The LC-MS analysis of JA and SA in plant extracts was performed at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University (SNU), Seoul, South Korea. Quantitation was performed with different concentrations of JA and SA.

Statistical analysis. For each experiment, leave and root samples were randomly collected from 10 plants. All the data obtained have mean values \pm SE. Measurements were performed on three replicates for each treatment ($n = 3$). The data was statistically analyzed by t-Student test to compare the differences between the means using the least significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

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Author contributions

B.A. conceived the original screening and research plans; M.A. and G.P. supervised the experiments; B.A., B.G. and M.A. performed most of the experiments; M.A. provided technical assistance to B.A.; B.A. designed the experiments and analyzed the data; E.H.C. conceived the project and wrote the article with contributions of all the authors; G.P. supervised and complemented the writing. E.H.C. agrees to serve as the author responsible for contact and ensures communication.

Competing interests

The authors declare no competing interests.

Additional information

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