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## RESEARCH ARTICLE OPEN ACCESS

# Plasma-Treated Water Induces Protein Oxidation in Mung Bean (*Vigna radiata*) and Chickpea (*Cicer arietinum*) Seedlings

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## ABSTRACT

Plasma-treated water (PTW) is a promising sustainable agricultural resource, yet its effects on seedling proteins remain unclear. This study quantified how plasma-derived reactive oxygen and nitrogen species modify proteins and influence early growth in chickpea and mung bean. Growth responses were species- and exposure-dependent ( $n = 3$ ): irrigation with PTW generated after 5 min of plasma exposure enhanced chickpea root growth, while 1-min PTW stimulated mung bean root elongation ( $p < 0.05$ ); however, longer exposures inhibited mung bean growth. Protein oxidation products analysis quantified *o*-tyrosine, *m*-tyrosine, 3,4-dihydroxyphenylalanine, dityrosine, dioxindolyl-L-alanine isomers, and 3-hydroxykynurenine. PTW irrigation increased some essential amino acids in chickpea seedlings, with methionine rising up to 75% compared to the control ( $p < 0.05$ ), and smaller increases in lysine and arginine contents.

## 1 | Introduction

Sprouts of various pulse crops are widely consumed across the world due to their high nutritional value [1]. Chickpea (*Cicer arietinum*) and mung bean (*Vigna radiata*) are among the most important food legumes, rich in essential amino acids, unsaturated fatty acids, dietary fibers, minerals, and bioactive compounds. These legumes have been staple foods for thousands of years, particularly in Asian countries [2]. However, increasing the productivity of major crops presents a substantial challenge, as global population growth—projected by the United Nations Food and Agriculture Organization (FAO) to exceed 9.7 billion by 2050—will further elevate the demand for such nutritious products, while available agricultural land continues to decline. Moreover, variability in seed coat properties, storage status, and microbial load can influence water uptake, uniformity, and early seedling vigor, thereby slowing

the establishment of the crop while increasing costs of production and maintenance [3]. To overcome these limitations, several approaches have been developed to enhance germination, including the application of exogenous phytohormones, chilling treatments, and scarification [4]. Nevertheless, these conventional methods are often time-consuming, labor-intensive, and may cause environmental pollution.

In recent decades, plasma and plasma-treated water (PTW), also referred to as plasma-activated water (PAW), have been widely gaining attention to enhance seed germination and plant growth owing to their cost-effectiveness, low energy consumption, and the absence of toxic residues [5, 6]. Moreover, in modern agriculture like hydroponic, which requires a continuous and sustainable supply of plant nutrients, PTW serves as a promising alternative or supplement to conventional fertilizers [7]. Plasma is a partially ionized gaseous state composed of

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ultraviolet (UV) photons, electrons, ions, free radicals, and reactive oxygen and nitrogen species (RONS) [8]. Among these reactive species, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide anion ( $\text{O}_2^{\cdot-}$ ), hydroxyl radical ( $\cdot\text{OH}$ ), ozone ( $\text{O}_3$ ), nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), and peroxyxynitrite ( $\text{ONOO}^-$ ) are considered the major reactive species responsible for its biological effects that can dissolve in water relatively [4]. In agricultural applications, nitrate holds particular importance because it serves as the main form of nitrogen absorbed by plants for the biosynthesis of amino acids, nucleotides, and various essential metabolites. In addition,  $\text{NO}_3^-$  is highly mobile within soil and exhibits greater stability than other nitrogen-containing ions (e.g.,  $\text{NO}_2^-$ ), making it a dependable and readily accessible nutrient source for plant growth [9]. While direct exposure to cold plasma can etch and modify the seed coat—thereby increasing its surface wettability and facilitating water uptake—PTW can exert similar or even greater effects, often resulting in significantly improved germination rates and accelerated seedling growth. Several studies have reported substantial improvements in germination rates and accelerated seedling development following plasma applications [10, 11]. However, these investigations often lack detailed mechanistic explanations for why germination is enhanced. Given that sprouts are frequently consumed directly, and PTW or plasma treatment is commonly applied to reduce surface microbial loads. Thus, it is essential to understand how plasma-derived species interact with seeds and germinating tissues that serve as food.

Proteins are essential biomolecules that play a key role in both food matrices and living systems, contributing significantly to nutritional value, flavor, and texture. The amino acid residues of proteins are particularly susceptible to oxidative modification, especially by RONS. Hydroxyl radicals ( $\cdot\text{OH}$ ), for instance, can react with amino acid side chains at near diffusion-controlled rates, with second-order rate constants ranging from approximately  $10^9$ – $10^{10} \text{ M}^{-1} \text{ s}^{-1}$  at physiological pH [12, 13]. Such oxidative reactions can alter the structural and functional properties of proteins [14, 15], reduce the bioavailability of essential amino acids, and may promote the formation of potentially harmful oxidation products. Numerous studies have demonstrated that cold plasma treatment of foods induces oxidative modifications in proteins and free amino acids [16]. While extensive research has focused on the effects of direct cold plasma treatment or PTW on seed germination and plant growth, as well as microbial disinfection [12, 13], relatively few investigations have examined its impact on specific chemical modifications of proteins in sprouts during their development. Moreover, most existing studies employ nonspecific analytical approaches, such as quantifying total protein carbonyls, to assess oxidation, which lacks molecular-level specificity and mechanistic insight [17]. Other studies report changes in total protein content and total sugar content following treatment [18, 19].

This study hypothesized that (i) irrigation of chickpea and mung bean seeds with PTW generated at different exposure durations would enhance seedling growth and sprout development, primarily due to the presence of RONS, like nitrate, in the PTW; (ii) PTW irrigation may induce moderate oxidative stress in seeds and/or sprouts, thereby triggering the biosynthesis of certain secondary metabolites, including amino acids, as part of

an antioxidant defense response; and (iii) PTW irrigation may cause chemical modifications in amino acid residues of seedling proteins, resulting in the formation of oxidation, nitrosation, and nitration protein products. Therefore, this study aims to identify and quantify oxidative, nitrate, and nitrosative products formed in chickpea and mung bean seedlings as a result of PTW irrigation, and to monitor changes in the amino acid composition of sprouts treated with PTW. To achieve this, the concentrations of major reactive oxygen and nitrogen species (RONS) generated in PTW by cold plasma treatment were first determined. Subsequently, stable oxidative end-products were identified and quantified using advanced liquid chromatography coupled with high-resolution Orbitrap mass spectrometry, while the amino acid composition of sprouts was analyzed by high-performance liquid chromatography with fluorescence detection (HPLC-FLD).

## 2 | Materials and Methods

### 2.1 | Materials and Chemicals

Mung beans and chickpeas were purchased from local Danish supermarkets (cultivar identity and seed history are not provided). *o*-Tyr ( $\geq 96.0\%$ ), *m*-Tyr ( $\geq 98\%$ ), L-3,4-dihydroxyphenylalanine (DOPA,  $\geq 98\%$ ), 3- $\text{NO}_2$ -Tyr ( $\geq 98\%$ ), 3-Cl-Tyr ( $\geq 97\%$ ), 5-OH-Trp ( $\geq 98\%$ ), kynurenine (Kyn,  $\geq 98\%$ ), kynurenic acid (KynA,  $\geq 98\%$ ), 3-OH-Kyn ( $\geq 98\%$ ), tryptamine (Tra,  $\geq 99\%$ ), methionine sulfoxide (MetSO,  $\geq 98\%$ ), methionine sulfone (MetSO<sub>2</sub>,  $\geq 98\%$ ), 4-nitro-L-phenylalanine monohydrate (4- $\text{NO}_2$ -Phe,  $\geq 98\%$ ), 5-nitro-DL-tryptophan (5- $\text{NO}_2$ -Trp,  $\geq 95\%$ ), 3,4-dehydro-DL-proline (3,4-DH-Pro,  $\geq 98\%$ ), N-nitrosoproline (NO-Pro,  $\geq 95\%$ ), amino adipic acid ( $\geq 98\%$ ), N-nitroso-N-methyl-DL-phenylalanine (N-NO-CH<sub>3</sub>-Phe,  $\geq 95\%$ ), 6-aminocaproic acid (ACA,  $\geq 99\%$ ), amino acid standards (500  $\mu\text{M}$ ), *o*-phthalaldehyde (OPA,  $\geq 99\%$ ), 3-mercaptopropionic acid (MPA,  $\geq 99\%$ ), sodium azide ( $\text{NaN}_3$ ,  $\geq 99.5\%$ ), sodium tetraborate decahydrate (99.5%–105.0%), trichloroacetic acid ( $\geq 99.0$ ), sodium borohydride ( $\text{NaBH}_4$ , 99%), hydrogen peroxide solution ( $\text{H}_2\text{O}_2$ , 30% w/w), hydrochloric acid (HCl, 37% w/w), methanol (HPLC-grade), formic acid (LC-MS grade), and acetonitrile (LC-MS grade) were provided from Sigma Aldrich (Darmstadt, Germany). *o,o*-Dityrosine dihydrochloride (DiTyr,  $> 99\%$ ) and dioxindolyl-L-alanine (mixture of diastereomers, DiOia-1 and DiOia-2) were sourced from Toronto Research Chemicals (Toronto, Canada). Tryptamine-*d*<sub>4</sub> HCl (Tra-*d*<sub>4</sub>, 99%) and *N*-formylkynurenine (NFK,  $\geq 98\%$ ) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). L-3,4-Dihydroxy-L-phenylalanine-*d*<sub>3</sub> (DOPA-*d*<sub>3</sub>, 98%), L-tryptophan-*d*<sub>5</sub> (Trp-*d*<sub>5</sub>, 98%), DL-3-hydroxykynurenine (3-OH-Kyn-<sup>13</sup>C<sub>3</sub><sup>15</sup>N, 95%), L-kynurenine sulfate-*d*<sub>6</sub> (Kyn-*d*<sub>6</sub>, 98%), *o,o*-dityrosine-<sup>13</sup>C<sub>2</sub> (diTyr-<sup>13</sup>C<sub>2</sub>, 99%), 3-chlorotyrosine HCl-<sup>13</sup>C<sub>9</sub><sup>15</sup>N (3-Cl-Tyr-<sup>13</sup>C<sub>9</sub><sup>15</sup>N, 98%), 3-nitro-L-tyrosine-<sup>13</sup>C<sub>6</sub> (3- $\text{NO}_2$ -Tyr-<sup>13</sup>C<sub>6</sub>, 99%), L-tyrosine-*d*<sub>7</sub> (Tyr-*d*<sub>7</sub>, 98%), L-phenylalanine-*d*<sub>2</sub> (Phe-*d*<sub>2</sub>, 98%), and kynurenic acid-*d*<sub>5</sub> (KynA-*d*<sub>5</sub>, 98%) were purchased from Cambridge Isotope Laboratories (MA, USA). Sodium nitrate ( $\text{NaNO}_3$ ,  $\geq 99\%$ ), sodium nitrite ( $\text{NaNO}_2$ ,  $\geq 97\%$ ), N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD,  $\geq 98\%$ ), sulfanilamide (SULF,  $\geq 99.5\%$ ), and vanadium(III) chloride ( $\text{VCl}_3$ ,  $\geq 97\%$ ), and potassium bis(oxalato)oxotitanate(IV) dihydrate ( $\geq 98\%$ ) were provided by Merck (Germany). Ultrapure water obtained from a Milli-Q® purification unit (Millipore, Bedford, USA) was used during the whole process.

## 2.2 | Plasma Setup and Seed Treatment Procedure

PTW was generated by expelling air plasma into ultrapure water. An atmospheric pressure plasma jet system (PlasmaBeam PC, Diener Electronic, Ebhausen, Germany) was used for this purpose, customized with a water-cooled condenser (4°C) installed at the nozzle to reduce the temperature of the expelled hot air plasma to room level. Pure compressed air (5 bar) with a flow rate of about 33 L/min served as the feed gas. The plasma source consisted of a central electrode (~2 mm in diameter) and a discharge chamber (opposite electrode/tube) with an approximate volume of 1 cm<sup>3</sup> and an inner diameter of ~5 mm (Diener Electronic, Ebhausen, Germany). All experiments were conducted under ambient temperature and humidity conditions. The discharged plasma was diffused directly into 50 mL of ultrapure water for 1, 5, or 10 min using a glass bubble diffuser with a pore size of 160–250 µm (the resulting water is referred to as 1-min PTW, 5-min PTW, and 10-min PTW, respectively, throughout the text). The pH of the resulting PTW samples was measured immediately after treatment using a pre-calibrated pH meter (Model 913, Metrohm, Germany).

Following plasma treatment, three Petri dishes were prepared, each containing three seeds with healthy morphology placed on filter paper (Whatman™, China), resulting in a total of nine seeds. Seeds were almost evenly spaced and placed on the filter paper to avoid contact between them and to ensure comparable hydration and oxygen availability. Untreated water (control) and PTW were sprayed onto the seeds using a manual sprayer at a distance of 20 cm, delivering approximately 2–3 mL per irrigation to ensure uniform droplet distribution. Petri dishes were sealed, incubated at 25°C, randomly positioned within the incubator, and repositioned daily. The irrigation process was repeated daily for 5 consecutive days. Chickpea and mung bean experiments were conducted concurrently. The PTW was freshly prepared for irrigation and used immediately.

## 2.3 | Quantification of Ozone (O<sub>3</sub>)

Indigo assay was used to quantify dissolved O<sub>3</sub> in a single biological replicate of PTW, according to the method described by Massima Mouele et al. (2020) [20], with measurements carried out in technical duplicate. In brief, stock solution A was prepared by dissolving 1.4 g of sodium hydrogen phosphate and 1.75 g of phosphoric acid (H<sub>3</sub>PO<sub>4</sub>, 85% w/w) in 50 mL of distilled water. Potassium indigotrisulfonate (1 M) was prepared in 0.1% (w/v) phosphoric acid and designated as stock solution B. To measure absorbance at 600 nm, 2 mL of each stock solution was transferred into a 25 mL volumetric flask. For the blank, the flask was filled to the mark with water, while for the sample measurement, PTW was used instead. The baseline of UV-Vis spectrophotometer was corrected (zeroed) using the stock solution A. The ozone concentration was determined using Beer-Lambert equation, applying a molar extinction coefficient of 22,000 M<sup>-1</sup> cm<sup>-1</sup>.

## 2.4 | Quantification of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) and Hydroperoxide (–OOH)

The concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroperoxides (–OOH) in a single biological replicate of PTW were

determined spectrophotometrically, with measurements carried out in technical duplicate. Hydrogen peroxide was quantified using a selective colorimetric method based on potassium bis (oxalato)oxotitanate(IV) dihydrate [21]. The titanium reagent (0.1 mM) was prepared in 5 M H<sub>2</sub>SO<sub>4</sub>, and 100 µL of PTW was mixed with an equal volume of 0.1 M NaN<sub>3</sub> in a cuvette. Subsequently, 100 µL of the titanium reagent was added, and the mixture was incubated for 10 min at room temperature before measuring the absorbance at 407 nm (LoQ of 5 µM).

The concentration of –OOH was quantified using the ferrous oxidation–xynol orange (FOX) assay [22]. This assay is highly sensitive (LoQ of 200 nM) but not selective. Therefore, it can quantify all types of peroxides in very low concentrations. Briefly, 50 µL of PTW was vortexed with 950 µL of freshly prepared FOX reagent, consisting of 9.8 mg ferrous ammonium sulfate, 7.6 mg xynol orange, and 1.82 g sorbitol in 100 mL of 25 mM H<sub>2</sub>SO<sub>4</sub>, and incubated in the dark at room temperature for 30 min. After incubation, absorbance of 150 µL of the mixture, which was transferred to a 96-well microplate was measured at 560 nm using a plate reader (SpectraMax i3x spectrophotometer, Molecular Devices, UK). The corrected absorbance was obtained by subtracting the reagent blank (50 µL untreated water and 950 µL FOX reagent) from the sample absorbance. The concentration of –OOH or H<sub>2</sub>O<sub>2</sub> was calculated from a standard calibration curve of H<sub>2</sub>O<sub>2</sub> prepared by diluting commercial H<sub>2</sub>O<sub>2</sub> in water.

## 2.5 | Quantification of Nitrate (NO<sub>3</sub><sup>–</sup>) and Nitrite (NO<sub>2</sub><sup>–</sup>)

Quantification of NO<sub>3</sub><sup>–</sup> and NO<sub>2</sub><sup>–</sup> in PTW was conducted by the Griess method from a single batch of PTW, with measurements carried out in technical duplicate. For NO<sub>3</sub><sup>–</sup> determination, 100 µL of appropriately diluted PTW was added to microplate wells, followed by an equal volume of VCl<sub>3</sub> solution (50 mM prepared in 1 M HCl). After mixing thoroughly, 50 µL of SULF (2% w/v prepared in 5% HCl) and 50 µL of aqueous solution of NEDD (0.1% w/v) were sequentially added as the Griess reagents. The microplate was incubated in the dark for 30 min, and absorbance was measured at 540 nm. NO<sub>2</sub><sup>–</sup> concentration was determined in a similar manner, except that VCl<sub>3</sub> was replaced with ultrapure water, and the PTW samples were directly reacted with the Griess reagents [23]. Subtraction of nitrite values from the nitrate values provides the correct concentration of nitrate. Calibration curves were constructed using sodium nitrate and sodium nitrite standards prepared in the range of 0–200 µM.

## 2.6 | CHNS Analysis

Protein content of dried seedlings was quantified using a CHNS analyser (vario MACRO cube, Germany) prior to subjecting the seedlings to acid hydrolysis. Nitrogen-to-protein conversion factors of 5.36 and 6.25 were used for chickpea and mung bean seedlings, respectively [24].

## 2.7 | Acid Hydrolysis of Seedlings

Acid hydrolysis of the seedlings was performed following the protocol described by Hawkins et al. (2009) under strictly

anoxic conditions [25]. Hydrolysis of seedling proteins into free amino acids is a prerequisite step for subsequent amino acid composition and protein oxidation product analyses. Briefly, the seedlings in each Petri dish were lyophilized using a freeze dryer (Lyovapor™ L-200, Germany), and the dried seedlings of each Petri dish were pooled and finely ground with a mortar and pestle, resulting in a total of three biological replicates ( $n = 3$ ). A known quantity of the resulting powder, corresponding to a defined protein content (at least 0.5 mg), was transferred into shell vials. Ultrapure water (200  $\mu$ L) was added to the samples, followed by the addition and vortexing of 25  $\mu$ L of deoxycholic acid (3 mg/mL), 10  $\mu$ L of sodium borohydride (1 mg/mL), and 50  $\mu$ L of ice-cold trichloroacetic acid (TCA, 50% w/v). The mixture was incubated at  $-20^{\circ}\text{C}$  for 10 min and subsequently centrifuged at 8000 g for 15 min. The resulting pellet was washed with pre-cooled TCA (5% w/v) and centrifuged again at  $5^{\circ}\text{C}$ . The washing procedure continued with ice-cold acetone for two additional cycles, each followed by centrifugation at 8,000 g for 5 min. The pellet was then dried under vacuum using a SpeedVac concentrator (Thermo Scientific, USA) to remove residual solvents. The dried pellet was hydrolyzed with 4 M methanesulfonic acid containing Tra (0.2% w/v) for 18 h at  $110^{\circ}\text{C}$  in Pico-Tag vessels. To prevent artefactual oxidative modifications during hydrolysis, samples were completely deoxygenated prior to heating by alternating high-purity nitrogen flushing and vacuum application for three cycles using a Waters Pico-Tag nitrogen workstation (Waters-PW-1650). The resulting hydrolysates were neutralized by adding an equal volume of freshly prepared 4 M NaOH and subsequently dried in the SpeedVac concentrator for 3 h. Finally, the dry hydrolysates were reconstituted in 0.5 mL of ultrapure water to obtain a known final concentration of seedling protein hydrolysates.

## 2.8 | Analysis of Amino Acid Composition and Met Oxidation Products

The amino acid composition and Met oxidation products, including MetSO and MetSO<sub>2</sub>, in chickpea and mung bean seedling hydrolysates (three biological replicates;  $n = 3$ ) were determined using an in-needle derivatization method in technical triplicate, automated through a Thermo Scientific Ultimate 3000 autosampler [26]. A final concentration of 100  $\mu\text{g/mL}$  of hydrolysates, along with 25  $\mu\text{M}$  of internal standard (ACA), was prepared and filtered through 0.22  $\mu\text{m}$  regenerated cellulose syringe filters (Phenomenex, Værløse, Denmark). The amino acids and Met oxidation products were derivatized by three reagents, which can be detected by an FLD at excitation and emission wavelengths of 340 nm and 440 nm, respectively. Reagent A was prepared using sodium tetraborate decahydrate (10 mM) adjusted to pH 10.2. Reagent B was a mixture of 20  $\mu\text{L}$  of OPA solution (7.5 mM) and 20  $\mu\text{L}$  of MPA (225 mM) diluted in 980  $\mu\text{L}$  of Reagent A, and 1 M of acetic acid was used as Reagent C. The derivatized samples were injected (10  $\mu\text{L}$ ) into an AdvanceBio AAA HPLC C18 column (2.7  $\mu\text{m}$ ,  $3 \times 100$  mm; Agilent, USA) set to  $40^{\circ}\text{C}$ . Aqueous mobile phase A was prepared by dissolving sodium phosphate dibasic anhydrous (9.86 mM), sodium tetraborate decahydrate (9.96 mM), and sodium azide (492  $\mu\text{M}$ ) in water, followed by adjustment of the pH to 8.2. Methanol, acetonitrile, and water with a ratio of 45:45:10 (v/v/v) were used as mobile phase B. The gradient program of elution

with a flow rate of 0.62 mL/min was as follows: 0–0.35 min, 2% B; 0.35–13.4 min, 2%–57% B; 13.4–13.5 min, 57%–100% B; 13.5–15.7 min, 100% B; 15.7–18 min, 2% B. A mixture of amino acid standards, MetSO, and MetSO<sub>2</sub> in the concentration range of 0–150  $\mu\text{M}$  was prepared to plot calibration curves.

## 2.9 | Analysis of Oxidation, Nitration, and Nitrosation Protein Products

Identification and quantification of specific oxidation, nitration, and nitrosation protein products in seedling hydrolysates (three biological replicates;  $n = 3$ ) was performed using an LC-Orbitrap MS/MS stable isotope assay in technical triplicate, following the protocol described in our previous publication [27]. Appropriately diluted seedling hydrolysates, along with the internal standard mixture, were filtered through 0.22  $\mu\text{m}$  regenerated cellulose syringe filters. The filtrates were placed in the autosampler set at  $10^{\circ}\text{C}$ . Each sample (10  $\mu\text{L}$ ) was injected into an Acquity UPLC HSS T3 column (100  $\text{\AA}$ , 1.8  $\mu\text{m}$ ,  $2.1 \times 100$  mm; Waters, Denmark) equilibrated at  $30^{\circ}\text{C}$  for chromatographic separation of analytes. The separation was achieved using two mobile phases: acetonitrile (mobile phase A) and 0.1% (v/v) aqueous formic acid (mobile phase B). Elution was performed using a linear gradient at a flow rate of 0.35 mL/min under the following conditions: 0–8.6 min, 100% B; 8.6–14.3 min, 0%–40% A; 14.3–16.8 min, 40%–60% A; 16.8–17.15 min, 60% A; 17.15–17.21 min, 60%–95% A; 17.21–18.6 min, 95% A; 18.6–18.64 min, 100% B; and 18.64–22.9 min, 100% B. Identification and quantification of targeted analytes were performed using an Orbitrap Exploris 480 high-resolution mass spectrometer (Thermo Scientific, USA) equipped with a heated electrospray ionization (H-ESI) source. The instrument was operated under standard MS tune parameters in positive ionization mode, with a spray voltage of 3.5 kV, a capillary temperature of  $320^{\circ}\text{C}$ , and a probe heater temperature of  $320^{\circ}\text{C}$ . Nitrogen was used as both the sheath gas (flow rate: 40 arbitrary units) and auxiliary gas (flow rate: 8 arbitrary units). Full MS scans were acquired over an  $m/z$  range of 70–800 with a resolving power of 17 500 FWHM, an AGC target of 3e6, and a maximum injection time of 100 ms. Quantification of analytes was carried out using parallel reaction monitoring (PRM) mode with a resolving power of 15 000 FWHM, an AGC target value of 2e5, a maximum injection time of 64 ms, and an isolation window of 2.0  $m/z$ . Chromatographic and mass spectrometric parameters for the stable isotope-labeled internal standards are summarized in Supporting Information S1: Table 1. Raw UHPLC-ESI-MS data were processed using Thermo Scientific FreeStyle™ software (version 1.8 SP2).

## 2.10 | Measurement of Seedling Length

From the onset of seedling growth (Day 2 of irrigation), the lengths of the two main seedling components—shoots (epicotyl and leaves) and roots (radicle and hypocotyl)—were determined for all seeds using a digital vernier caliper (Tesa Eagle, Switzerland). Measurements were performed daily by the fourth day of seedling growth (Day 5 of irrigation).

## 2.11 | Statistical Analysis

GraphPad Prism 10.5 (GraphPad Software, USA) was used for statistical analyses. Three biological replicates were conducted ( $n = 3$ ), and each biological replicate was analyzed in technical triplicate unless otherwise stated. Values are presented as means  $\pm$  standard deviation/standard error of the mean. One-way ANOVA was employed for all other datasets. Differences among group means were assessed using Dunnett's multiple comparison test. Statistical significance was defined at a confidence level of  $\alpha = 0.05$ , with significance levels denoted as follows: \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

## 3 | Results and Discussion

Mung bean and chickpea seeds were irrigated with PTWs generated at different cold plasma exposure times, while ultrapure water served as the control irrigation. The choice of ultrapure water as control and for preparation of PTW enables controlled mechanistic investigation of plasma-generated RONS and their direct effects on seedling proteins. The absence of ions, buffering reagents, and minerals minimizes secondary reactions and allows clearer interpretation of RONS-driven chemistry. However, agricultural irrigation water contains metal ions that can influence the distribution of RONS, their lifetime, and reactivity. Prior to application, it was essential to quantify the formation of stable RONS in the PTWs to ensure a precise understanding of their chemical composition and potential biological effects. Ultrapure water was used since it contains negligible amounts of organic compounds and minerals, thereby allowing accurate screening of plasma-generated reactive species without interference or scavenging by extraneous constituents.

### 3.1 | Quantification of Reactive Species in PTW

The pH and RONS data presented here were obtained from our previous publication [16], conducted under the same plasma treatment conditions (see Supporting Information S1: Figure 1). The pH of PTW decreased gradually from approximately 6.5 to 3 with increasing plasma exposure duration [16]. This pH drop and acidification are likely attributed to the formation of nitric acid and nitrous acid in the aqueous phase, resulting from oxidative reactions of RONS upon plasma discharge [28]. Conductivity and titratable acidity of PTW were not measured in the present study. Though  $\cdot\text{OH}$  is known to be formed during plasma treatment, it could not be quantified due to its extreme reactivity and short lifetime [12, 13, 29]. However,  $\cdot\text{OH}$  can recombine to generate  $\text{H}_2\text{O}_2$  [30]. Irrigation with PTW containing  $\text{H}_2\text{O}_2$  can regulate transcriptional processes in seeds and seedlings, including the synthesis of antioxidant enzymes and phytohormones such as abscisic acid, gibberellic acid, brassinosteroids, auxin, and ethylene [31]. Nevertheless, we observed no  $\text{H}_2\text{O}_2$  in PTW [16] as also observed previously by Pandey et al. (2016) [32]. The generated  $\text{H}_2\text{O}_2$  could degrade due to the reaction between  $\text{NO}_2^-$  and  $\text{H}_2\text{O}_2$  under acidic conditions [33]. The ferrous oxidation–xylenol orange (FOX) assay revealed the presence of hydroperoxides ( $-\text{OOH}$  species) in the range of 4–7  $\mu\text{M}$  (Supporting Information S1: Figure 1,  $p < 0.001$ , Dunnett's test). PTW also contained  $\text{O}_3$ , which is

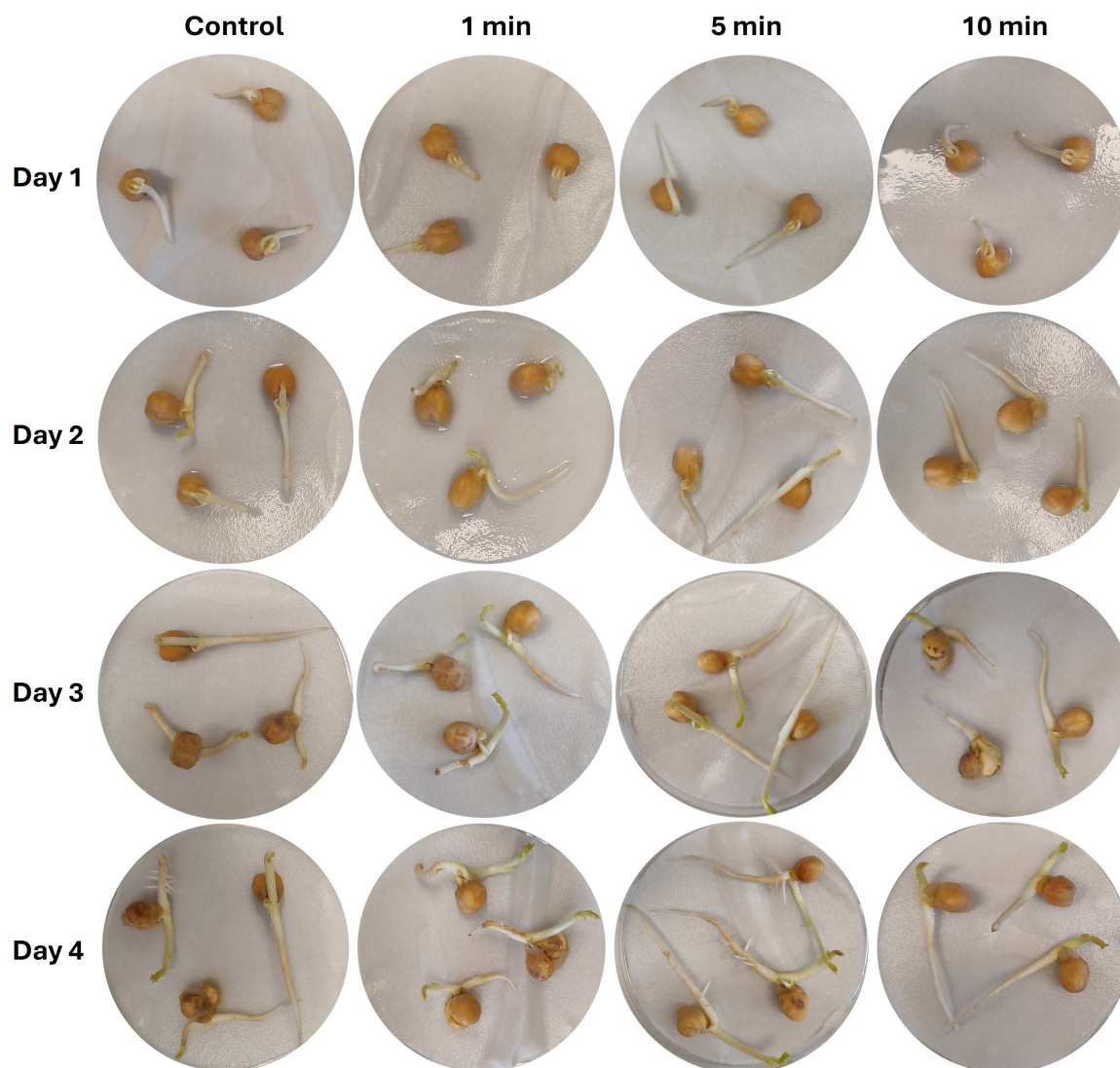
known to be highly reactive [34, 35], in the range of  $10.8 \pm 0.0 \mu\text{M}$  to  $23.45 \pm 0.1 \mu\text{M}$  [16]; though much lower than those reported in a study using dielectric barrier discharge (DBD) plasma, which generated 0.5 mM  $\text{O}_3$  at pH 2.5 after 5 min of exposure [20]. The presence of nitrogen in plasma feed gas promotes the formation of  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , which are known for their fertilizer role in agricultural applications. The concentration of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in PTW increased progressively ( $p < 0.001$ , Dunnett's test) with prolonged exposure, reaching approximately 1.8 mM and 800  $\mu\text{M}$ , respectively, after 10 min of treatment (Supporting Information S1: Figure 1C, D) [16]. Overall, the RONS composition of the PTW was anticipated to modulate protein oxidation and seedling growth upon irrigation, as detailed in the subsequent sections.

### 3.2 | Effect of PTW on Seedling Growth

Chickpea seeds were soaked in distilled water overnight to ensure seed viability and promote uniform seedling growth. After soaking, seeds with healthy morphology and intact appearance were selected for further use [36]. In contrast, mung beans were not subjected to soaking due to their naturally high germination rate and rapid sprouting ability. Following selection, three seeds were placed on a piece of filter paper within each Petri dish (i.e., three Petri dishes and each contains three seeds). Irrigation was performed by spraying either untreated water (control) or PTW, the latter prepared by exposing water to plasma for durations of 1, 5, and 10 min.

The measurements of the seedlings were divided into two categories: (i) root growth, which included the radicle and hypocotyl during early development, and (ii) shoot growth, comprising the epicotyl and leaves. Seedling development under irrigation with PTWs over 5 days is presented in Figure 1 and Figure 2 for chickpeas and mung beans, respectively.

As shown in Figure 3A, root elongation was greatest in chickpea seedlings treated with 5-min PTW, exhibiting slightly higher growth compared to those irrigated with untreated water (control). This enhancement may be attributed to the presence of  $\text{NO}_3^-$  in PTW, which can serve as a nitrogen source, inactivating seed diseases, and enhancing antioxidant defense mechanisms in plants, thus stimulating early root development [18]. Moreover, endogenously generated ROS from mitochondria in the seedlings, together with exogenous ROS introduced by PTW, act synergistically to accelerate growth via activating catalase-encoding genes [37]. However, irrigation with 10-min PTW, which contained higher concentrations of nitrates and other RONS, resulted in a reduced seedling growth compared to the control. This reduction in growth may be attributed to the excessive accumulation of reactive species, such as  $\text{NO}_2^-$ , which at elevated levels can exert phytotoxic effects and trigger oxidative or nitrosative stress in seedlings [38, 39]. Alternatively, overstimulation by RONS may activate a broad spectrum of stress-related pathways through the upregulation of defense and repair gene expression [7]. Shoot development of chickpea seedlings followed a similar trend to root growth (Figure 3B). The emergence of the first leaves was observed on the third day of irrigation (Day 2 of seedling growth). Among the treatments, seedlings irrigated with 5-min PTW exhibited the most



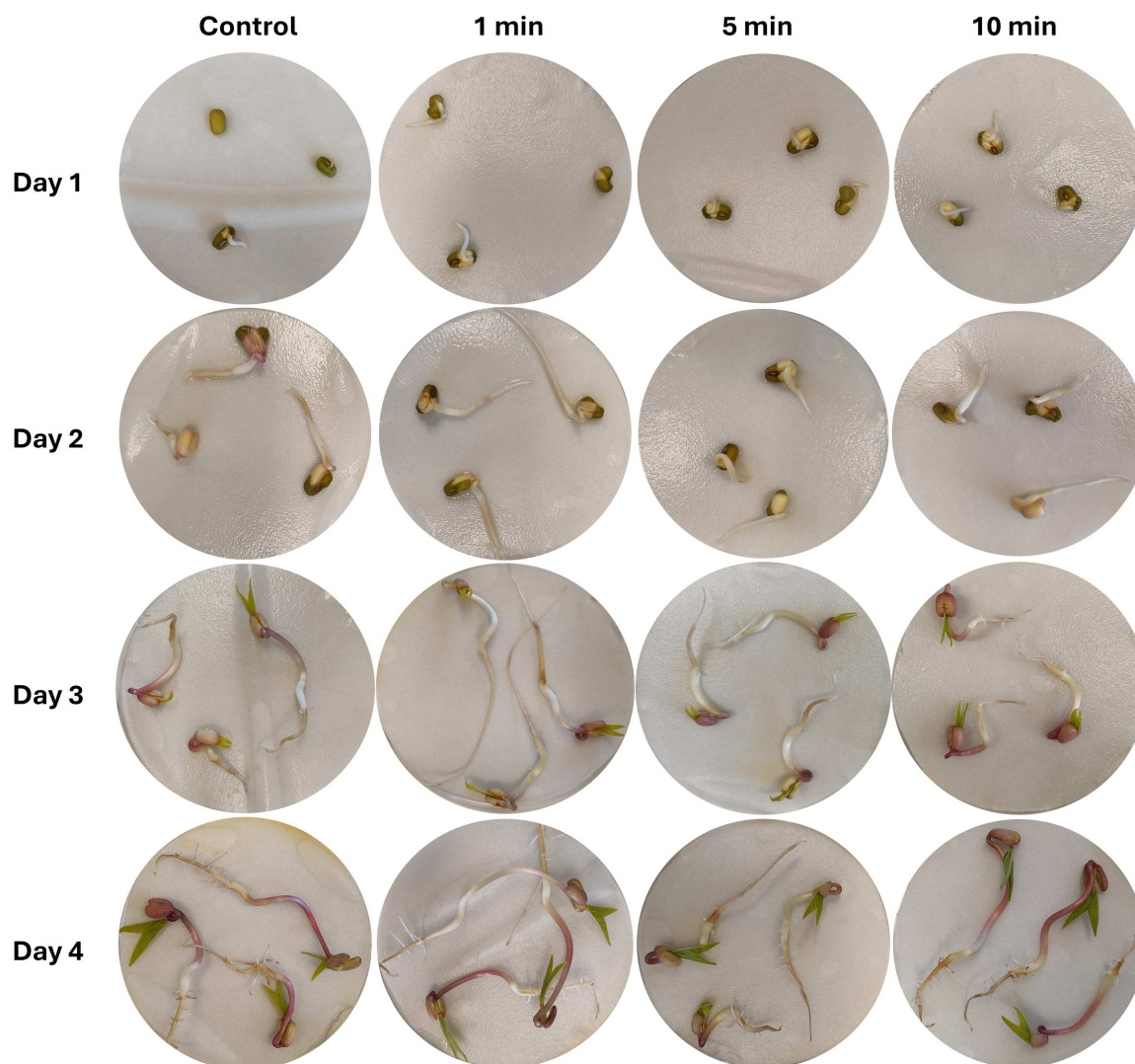
**FIGURE 1** | Germinated chickpeas irrigated with untreated water and PTW over a 5-day period (excluding day zero). Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min.

pronounced shoot elongation and leaf development ( $p < 0.05$ , Dunnett's test). In contrast, irrigation with 10-min PTW significantly inhibited shoot growth ( $p < 0.001$ , Dunnett's test), compared to both the control and shorter plasma treatment durations.

Root development in mung beans was first observed on the second day of irrigation (Figure 3C). Seedlings treated with 1-min PTW exhibited the most pronounced root elongation ( $p < 0.001$ , Dunnett's test), followed by those irrigated with the control, 10-min PTW, and 5-min PTW. However, these differences became evident only after the fourth day of irrigation (Day 3 of seedling growth). Shoot emergence occurred on the fourth day, with 1-min PTW again promoting greater shoot growth compared to the control and other treatments (Figure 3D). By the fifth day, however, the differences among treatments were not statistically significant, suggesting that the stimulatory effects of PTW were transient and diminished as the seedlings continued to develop.

### 3.3 | Formation of Advanced Oxidation, Nitration, and Nitrosation Protein Products

Both untreated and PTW-irrigated seedlings, along with their corresponding seeds (i.e., cotyledons) after 5 days of irrigation, were freeze-dried and subsequently subjected to acid hydrolysis. The protein content of freeze-dried samples was measured using CHNS method. Chickpea and mung bean seedlings had 25% and 36% protein, respectively. The resulting hydrolysates were then analyzed for the identification and quantification of protein oxidation, nitrosation, and nitration products. The targeted products are summarized in Supporting Information S1: Table S1. Notably, no nitrated or nitrosated products were identified in the PTW irrigated seedlings, in contrast to when free amino acids or protein solutions were treated by cold plasma as studied in our previous findings [15, 16]. This absence may be attributed to the metabolic activity of the plant or the presence of endogenous antioxidant defense molecules and enzymes that mitigate direct nitrosative modification of seed proteins [40].



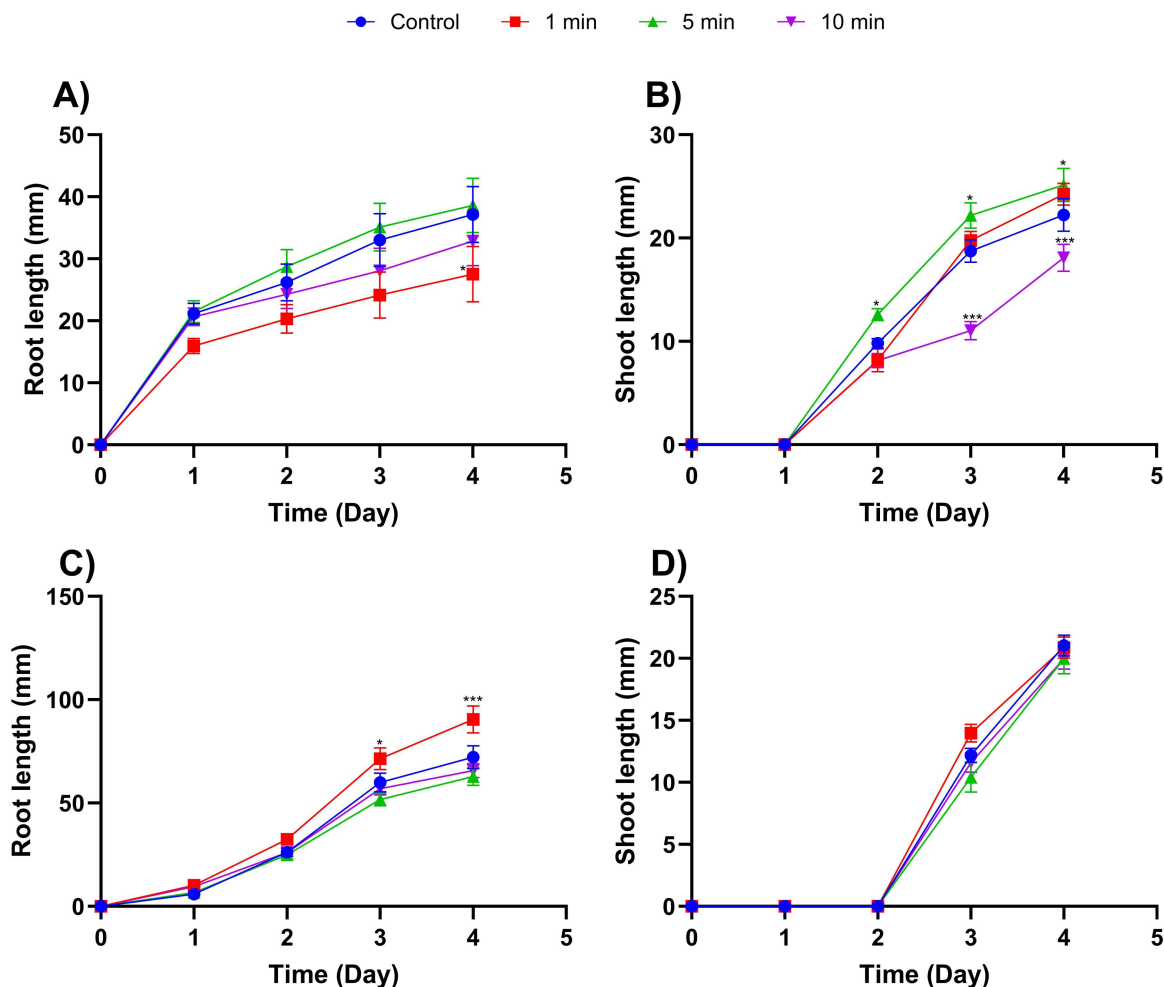
**FIGURE 2** | Germinated mung beans irrigated with untreated water and PTW over a 5-day period (excluding day zero). Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min.

In this study, oxidation markers were measured after 5 days of irrigation, and the concentration of oxidation products in dry seeds or early germination time points was not included. When evaluated for protein oxidation products, for both chickpea and mung bean seedlings, Phe, Tyr, and Trp oxidation products, including *o*-Tyr, *m*-Tyr, DiTyr, DOPA, DiOia-1/–2, and 3-OH-Kyn, were identified and subsequently quantified in a range of ng/g seedlings protein. Quantification of specific cysteine oxidation products, such as cystine and cysteic acid, was not feasible because cysteine undergoes artifactual oxidation during acid hydrolysis, leading to biased results [25]. Furthermore, the determination of certain oxidation markers, including 2-oxo-histidine, was not possible due to the unavailability of authentic standards.

Irrigation of chickpeas with PTW for 10 min resulted in an increase in the formation of *o*-Tyr and *m*-Tyr by approximately 40 ng/g of chickpea protein (Figure 4A–B). Similarly, the concentration of DOPA increased to nearly 70 ng/g of protein, compared with 32 ng/g in chickpeas irrigated with untreated water. In contrast, prolonging the PTW treatment time led to a gradual decrease in the concentration of DiTyr by about 3 ng/g

of protein, suggesting further modification or degradation of this oxidation product (Figure 4C). A similar trend was observed for 3-OH-Kyn. Conversely, irrigation with PTWs steadily increased the formation of DiOia-1/–2, although a reduction in these compounds was noted in chickpeas irrigated with 10-min PTW compared with the control samples. This could be due to further degradation to other compounds like diol [41]. Estévez et al. (2017) suggested that dietary exposure to certain oxidation products could, in principle, contribute to health risks under high or chronic exposure [42, 43]. Accumulation of DiTyr in the body could enhance generation of ROS, thereby exacerbating oxidative stress and chronic inflammation [44]. Moreover, in vivo protein oxidation has been mechanistically linked to processes involved in the pathogenesis of various neurodegenerative disorders, including Alzheimer's disease [45], but the relevance of these findings at typical dietary exposure levels remains speculative and requires further study.

Although the formation of DiOia-1 and DiOia-2 in mung beans exhibited a trend comparable to that observed in chickpeas, their concentrations were approximately threefold higher



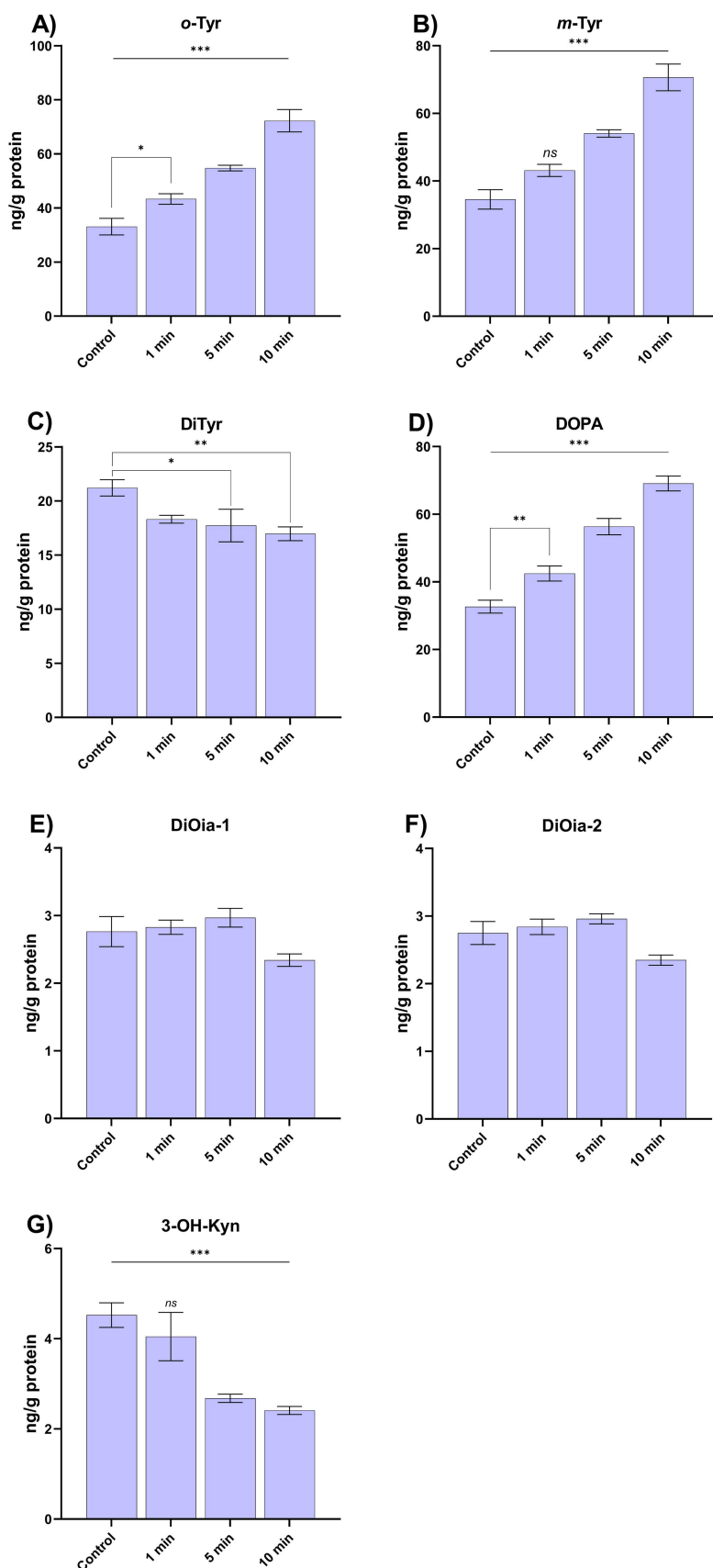
**FIGURE 3** | Root and shoot length measurement of the chickpeas (A and B) and mung beans (C and D) irrigated with untreated water and PTW over a 5-day period (excluding day zero). Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min. Statistically significant differences between the treated and control samples are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (one-way ANOVA followed by Dunnett's test,  $\alpha = 0.05$ ).

(Figure 5). In addition, the formation of 3-OH-Kyn increased by approximately 4 ng/g of mung bean protein, when seeds were irrigated with 5-min PTW. In contrast, the oxidation products derived from Phe and Tyr exhibited different trends compared to those observed in chickpeas. Irrigation of mung beans with 1-min PTW led to an increase of approximately 50 ng/g of *o*-Tyr and *m*-Tyr, a rise comparable to that observed in chickpeas irrigated with 10-min PTW. However, irrigation with 5- or 10-min PTW did not further increase the contents of these oxidation products. Similar trends were also observed for DiTyr and DOPA (Figure 5). Induced protein oxidation and nitration exert a dual regulatory influence on seedling growth. Low levels of specific reactive species, such as  $H_2O_2$  or nitric oxide (NO), can promote seedling growth and maintain seed viability by functioning as redox signals [46–48]. These species modulate the activity of key metabolic enzymes, facilitate the mobilization of storage proteins, and regulate hormonal pathways that control the transition from dormancy to germination. In particular, NO has been reported to delay the loss of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), thereby stabilizing the cellular redox environment during early imbibition [47]. Conversely, excessive or non-selective oxidation or nitration can impair seedling establishment.

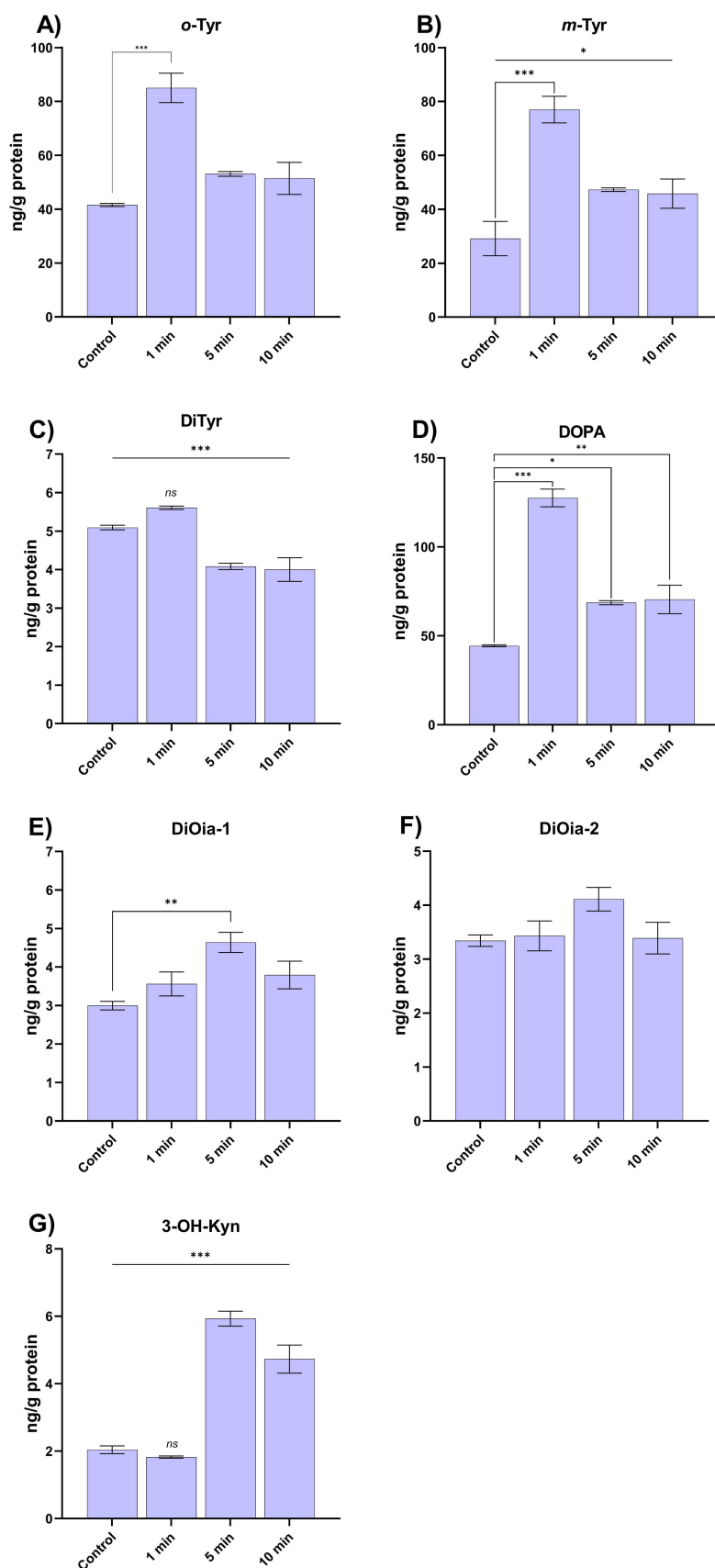
Overexposure to PTW may cause loss of specific proteins and enzymes' function and integrity, which reduces seedling growth.

### 3.4 | Changes in Amino Acid Profile of Chickpea and Mung Bean Seedlings

Irrigation of seedlings with PTW containing RONS may either induce oxidative stress that stimulates the synthesis of specific amino acids or promote the oxidation of seedling proteins. Quantitative assessment of these amino acid losses offers valuable insights into the nutritional implications of PTW treatment, as the formation or loss of essential amino acids directly affects overall protein quality. The hydrolysates obtained from acid hydrolysis of seedlings were appropriately diluted and injected into an HPLC-FLD system along with an internal standard (ACA). Amino acids were derivatized using OPA/MPA and subsequently separated by reverse-phase chromatography. Amino acids such as Asn, Gln, and Cys undergo artifactual deamidation and oxidation during acid hydrolysis and, therefore, cannot be accurately quantified using this method. Consequently, Asx represents the



**FIGURE 4** | Concentration of different protein oxidation products (A–G) formed in chickpeas irrigated by untreated water and PTW. Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min. Statistically significant differences between the treated and control samples are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (one-way ANOVA followed by Dunnett's test,  $\alpha = 0.05$ ). The mean values are displayed as bar graphs in the corresponding figure.



**FIGURE 5** | Concentration of different protein oxidation products (A–G) formed in mung beans irrigated by untreated water and PTW. Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min. Statistically significant differences between the treated and control samples are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (one-way ANOVA followed by Dunnett's test,  $\alpha = 0.05$ ). The mean values are displayed as bar graphs in the corresponding figure.

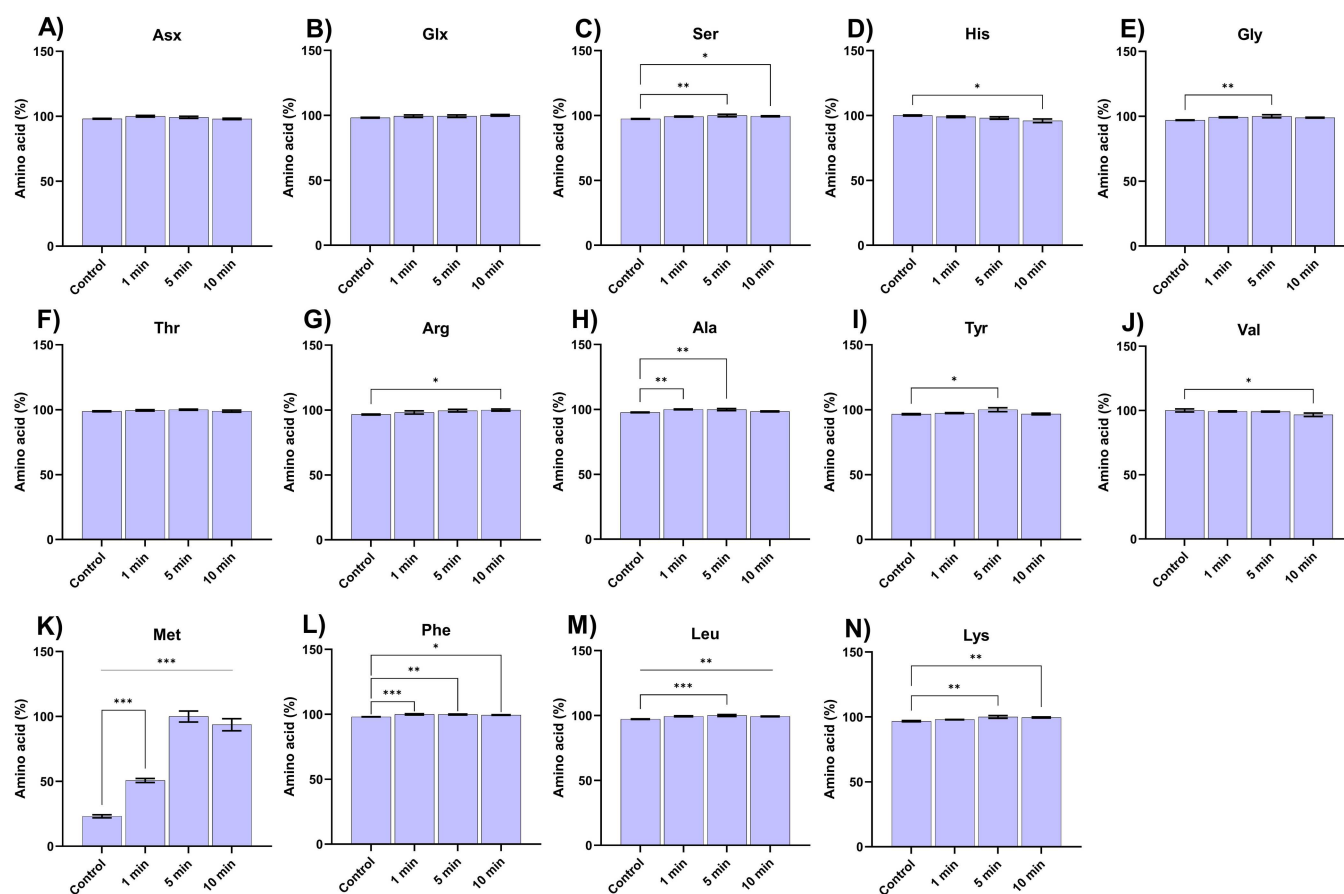
combined content of Asp and Asn, whereas Glx represents the combined content of Glu and Gln. Changes in the amino acid profiles of chickpeas and mung beans under different treatments are presented in Figures 6 and 7, respectively. The concentrations (mg/g) of individual amino acids in chickpea (Supporting Information S1: Figure 2) and mung bean (Supporting Information S1: Figure 3) seedling proteins are presented in the Supplementary.

Figure 6 shows that the His and Val content decreased by approximately 5% ( $p < 0.05$ , Dunnett's test) in chickpeas irrigated with 10-min PTW, consistent with its known susceptibility to oxidation. Interestingly, most other amino acids, despite being prone to oxidation, exhibited the opposite trend, with increased concentrations, which may be attributed to stress-induced metabolic responses in the seedlings, leading to enhanced synthesis of these metabolites. Met content, for example, significantly increased by 25% and 75% ( $p < 0.001$ , Dunnett's test) when chickpeas were irrigated with 1-min and 5-min PTW, respectively. Given that Met is a limiting sulfur-containing amino acid in many plant-based foods, such enhancement suggests a promising strategy to improve its nutritional contribution.

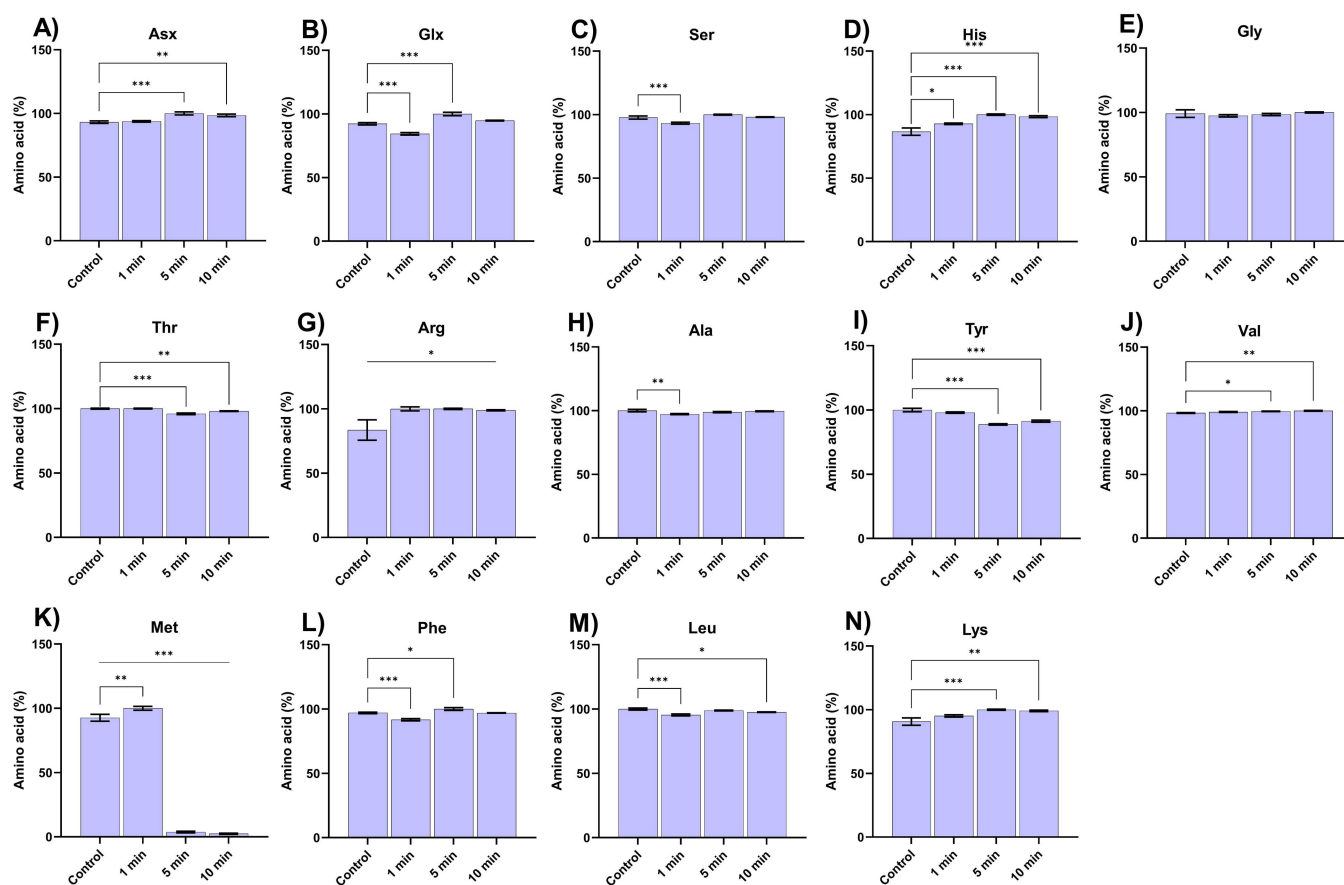
In contrast, the amino acid profile of mung beans (Figure 7) exhibited trends different from those observed in chickpeas (Figure 6). His and Val increased following PTW irrigation. Met increased by ~8% ( $p < 0.01$ , Dunnett's test) with 1-min PTW but decreased sharply by ~90% ( $p < 0.001$ , Dunnett's test) under 5- or 10-min PTW. Other amino acids, including Thr, Tyr, and Leu, declined with longer treatment durations. Interestingly, for Glx (Glu + Gln), Ser, Phe, and Leu, 1-min PTW caused significant decreases, whereas 5-min PTW reversed this effect, leading to increased concentrations. In both chickpea and mung bean seedlings, Trp was not quantified because its concentration was below the limit of quantification. The content of Arg and Lys, the latter being an important essential amino acid, increased slightly in both species when irrigated with PTW (Figures 6–7). These results indicate species-specific amino acid responses to PTW-induced stress, with both increases and decreases reflecting complex metabolic adjustments in response to oxidative stimuli.

### 3.5 | Study Limitations

A few limitations of the current study should be considered when interpreting the results. The sample size was modest (nine seedlings per treatment), and the experiments were conducted under



**FIGURE 6** | Change in amino acid profile (A–N) of chickpeas after irrigation with PTW. Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min. Concentration of the amino acid in the control (irrigated with untreated water) sample was represented as 100%, and changes in concentration of treated samples (irrigated with PTW) were represented relative to the control sample. Statistically significant differences between the treated and control samples are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (one-way ANOVA followed by Dunnett's test,  $\alpha = 0.05$ ). The mean values are displayed as bar graphs in the corresponding figure.



**FIGURE 7** | Change in amino acid profile (A–N) of mung beans after irrigation with PTW. Control, 1 min, 5 min, and 10 min correspond to irrigation with untreated water (control) or PTW generated by plasma exposure for 1, 5, or 10 min. Concentration of the amino acid in the control (irrigated with untreated water) sample was represented as 100%, and changes in concentration of treated samples (irrigated with PTW) were represented relative to the control sample. Statistically significant differences between the treated and control samples are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (one-way ANOVA followed by Dunnett's test,  $\alpha = 0.05$ ). The mean values are displayed as bar graphs in the corresponding figure.

controlled laboratory conditions using Petri dish and nutrient-free Milli-Q water, which do not fully replicate soil environments. As reported in Section 3.1, PTW exhibited a reduced pH (to  $\sim 3$ ) and contained measurable concentrations of nitrate ( $\sim 1.8$  mM) and nitrite ( $\sim 0.8$  mM). In the absence of pH-matched controls (e.g., acidified water) and nitrogen-equivalent controls, it is not possible to fully distinguish between the effects of acidification, nitrogen supplementation, and plasma-generated RONS. Therefore, the observed biological responses should be interpreted as the combined effect of these factors rather than being attributed solely to plasma-specific mechanisms. Furthermore, oxidation markers were assessed at a single time point (last day of seedling growth) due to limited biomass available for LC-Orbitrap MS/MS analysis. Moreover, molecular signaling analyses were not conducted to determine the underlying mechanisms of PTW-induced changes in seed germination. Therefore, the results should be interpreted as indicative of early-stage responses in a controlled model system rather than as long-term agronomic or health-related outcomes.

#### 4 | Conclusion

This study evaluates the physicochemical properties of PTWs generated under varying cold plasma exposure durations and their

subsequent effects on seedling growth, protein oxidation, and amino acid metabolism in chickpea and mung bean seedlings. Seedling growth experiments demonstrated that PTW can have different effects on different seed types and different parts, including roots and shoots. The moderate PTW may promote early root and shoot development, whereas prolonged treatment inhibits growth. PTW irrigation induced the formation of several oxidative markers, such as *o*-Tyr, *m*-Tyr, DOPA, DiTyr, DiOia-1/-2, and 3-OH-Kyn, while nitrated or nitrosated products were absent. The extent and pattern of these oxidation products varied between chickpea and mung bean seedlings, reflecting species-dependent oxidative resilience and metabolic regulation. PTW irrigation alters nitrogen and sulfur. Chickpea seedlings display enhanced levels of several amino acids under moderate PTW exposure, suggesting activation of adaptive biosynthetic pathways under mild oxidative stress. In contrast, mung bean seedlings exhibited significant depletion of sulfur- and aromatic-containing amino acids under prolonged exposure. Therefore, optimization of cold plasma in generating PTW can help to balance RONS molecules, which are critical for seedling development, as excessive RONS may inhibit germination. Finally, the mechanistic insights provided in this study help clarify why and how plasma-generated water influences early plant development and offer basic knowledge for tailoring PTW for irrigation applications.

## Author Contributions

**Mohammad Bayati:** conceptualization, writing – original draft, software, methodology, investigation, formal analysis, data curation. **Brijesh K. Tiwari:** conceptualization, review and editing. **Mahesha M. Poojary:** conceptualization, writing – review and editing, supervision, resources, project administration, methodology, funding acquisition, data curation.

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## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supporting File:** ppap70189-sup-0001-Supplementary\_R1.docx.