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Effects of Nonthermal Plasma (NTP) on the Growth and Quality of Baby Leaf Lettuce (*Lactuca sativa* var. *acephala* Alef.) Cultivated in an Indoor Hydroponic Growing System

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Abstract: The aim of this research was to develop an effective protocol for the application of nonthermal plasma (NTP) technology to the hydroponic nutrient solution, and to investigate its effects on the growth and quality of baby leaf lettuce (*Lactuca sativa* var. *acephala* Alef.) grown in a hydroponic growing system (HGS) specifically designed for indoor home cultivation. Four HGSs were placed in separate growth chambers with temperature of 24 ± 1 °C and relative humidity of 70 ± 5%). Lettuce plants were grown for nine days in nutrient solutions treated with NTP for 0 (control) to 120 s every hour. Results of the first experiments showed that the optimal operating time of NTP was 120 s h^{-1}. Fresh leaf biomass was increased by the 60 and 120 s NTP treatments compared to the control. Treating the nutrient solution with NTP also resulted in greater leaf content of total chlorophylls, carotenoids, total phenols, and total antioxidant capacity. NTP also positively influenced chlorophyll *a* fluorescence in Photosystem I (*PSI*) and photosynthetic electron transport. These results revealed that the NTP treatment of the nutrient solution could improve the production and quality of hydroponically grown baby leaf lettuce.

Keywords: antioxidants; chlorophyll *a* florescence; eustress; floating system; hydroponics; nonthermal plasma treatment; nutrient solution

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1. Introduction

Nonthermal plasma (NTP) is ionized gas created during a plasma discharge at the gas–liquid interface. This process generates a mixture of reactive oxygen and nitrogen species (ROS and RONS) with final production of nitrate, hydrogen peroxide, and ozone, which can all influence plant physiology and growth [1].

Nonthermal plasma is an emerging technology for agricultural sector that has recently gained considerable interest for application in the agrifood industry [2,3]. Reactive particles from plasma can oxidize molecules in the extracellular space or lipids in the cytoplasmic bilayer. The latter could result a possible route for RONS in the creation of nanopores to enter the cell and oxidize molecules in the cytoplasm [4].

Particles outside the cell can also interact with appropriate receptors. Oxidized molecules or membrane lipids and activated receptors can all transduce a signal about the increased amount of RONS, and the cell can react by changes in gene expression, the activation of defense mechanisms, or signal transduction to other cells [4].

Among the first applications of NTP on crop production, there are treatments for seed disinfection and priming [5–7]. Seed germination and seedling establishment are favorably

influenced by NTP treatment [6]; although the mechanisms involved are not completely clear, they seem to be associated with greater water uptake and the removal of microbial layers on seed surface. Arc et al. (2013) [8] reviewed the role of nitric oxide (NO) in seed germination and plant growth, and correlated the role of NO and ROS species on the positive effect of abscisic acid (ABA) production, which is the pivotal hormone responsible for the ignition and maintenance of seed dormancy [9].

Positive effects on seed germination and plant development were found in hemp, radish seeds, tomatoes, and peppers treated with NTP [9–11].

A considerable number of studies have been published on the NTP effects on seed germination and seedling development, but only few investigated the application of NTP on the growth and development of adult plants [4]. For instance, the application of water or air treated with NTP to tomato plants increased fruit number and size, and flavonoid content, and reduced the pH of fruit juices [12]. NTP treatment promoted nutrient uptake, plant growth, and flower production in gerbera grown in peat [13]. In peppers, root and shoot growth was stimulated by treatments with NTP [14]. Recent evidence suggested that NTP is an effective physical disinfection method against pathogens such as *Fusarium oxysporum* [15], and can alleviate the negative effects of salinity stress [16] and exposure to zinc oxide or selenium nanoparticles [17].

Plasma-activated water (PAW) creates an acidic environment that results in changes in redox potential and electrical conductivity, and in the formation of ROS and RONS species with final production of ozone, hydrogen peroxide, and nitrate [1,18]. However, the effects of different types of plasma on chemical composition of various types of water are different [1]. Several studies demonstrated the benefit of PAW for plants grown in hydroponics or in soilless media [1,18]. However, most of these studies used water without additional fertilizer as a control treatment, and this can explain the growth-stimulating effects due to the synthesis of nitrogen compounds. The effect of NTP treatment on nutrient composition in hydroponic solutions is unknown [19].

There is a growing interest for the greenhouse or indoor production of vegetables [20]. Indoor (or vertical) farming is a novel cultivation concept that aims to increase plant productivity per unit area of cultivated land and is based on the use of LED lighting [21]. Home gardening is also globally growing through all levels of society and is the most common form of urban agriculture [22]. Accessibility to fresh, healthy, and cheap food is one of the main reasons of interest in both indoor horticulture and home cultivation.

The general objective of the present study was to develop an effective protocol for NTP treatment of a nutrient solution in hydroponic (floating system) cultures performed in hydroponic growing system unit (HGS) that was specifically designed for indoor home cultivation. The HGS was equipped with a NTP generator that insufflated ionized air into the nutrient solution.

The work was carried out on baby leaf lettuce. The market of freshly cut baby leaves increased over the last 20 years in Europe [23]. Lettuce is the most popular leafy vegetable in salads and, due to its high content in carotenoids and phenolics, it can provide a significant proportion of the content of total antioxidants in the diet [24].

Different experiments were carried out with the aim to: (i) characterize the correct operation of the NTP system developed for the indoor cultivation; (ii) determine the optimal working time of the NTP system to avoid any damage to plant roots and leaves; and (iii) evaluate the effects of NTP treatment on the ion composition of the nutrient solution, and on the production and quality of baby leaf lettuce.

2. Materials and Methods

2.1. NTP Generator Description

NTP refers to a process involving plasma formation during which electron energy and temperature reach 1–20 eV. Many kinds of particles, such as ions, excited atoms, molecules, and free radicals are generated at lower temperatures (close to room temperature).

Plasma generators used in this work were designed, developed, and manufactured by Jonix SpA (Padova, Italy; https://jonixair.com/ last accessed the link: 11 March 2022). They were dielectric barrier discharge (DBD) reactors consisting of a quartz cylinder coated with a metal mesh (exposed electrode) in which there were numerous holes and an electrode placed inside (encapsulated ground electrode) (Figure 1). The system was powered by electric current; following the electric discharge that is generated, electrons accumulating on the metal mesh where the gaseous flow that laps against this surface are then ionized to form cold plasma (Figure 1).

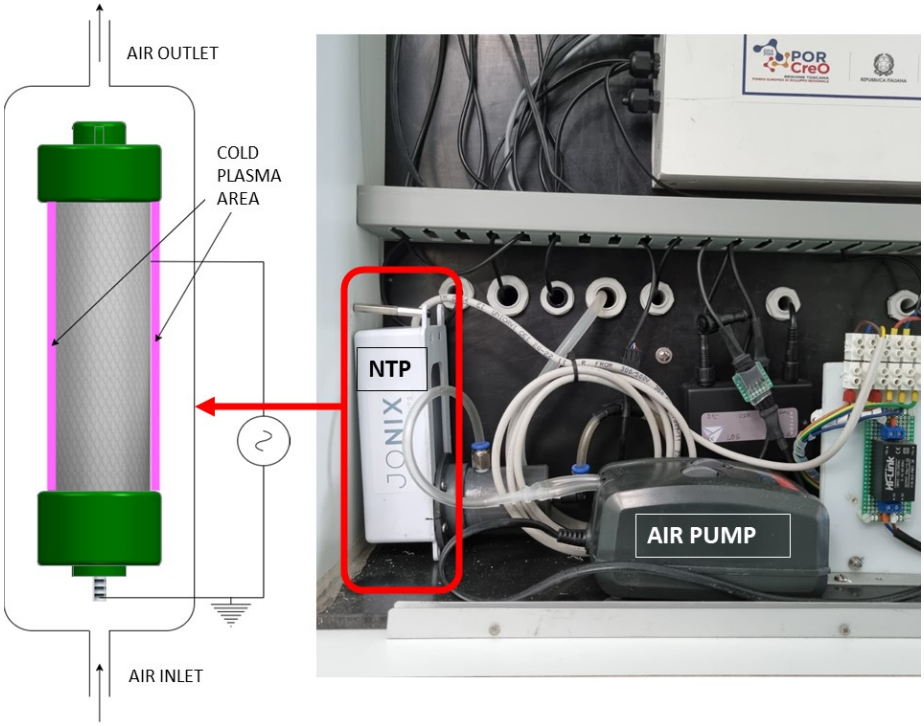

Figure 1. Nonthermal plasma (NTP) system installed inside hydroponic growing system (HGS) for home plant cultivation. Ionized air flow bubbles in the nutrient solution in floating system installed inside HGS.

The NTP generator used in the experiments was a small device consisting of a dielectric barrier discharge (DBD) plasma reactor 4 cm long and 1 cm in diameter, powered at 50 Hz frequency and 2 kV tension provided by a custom high-voltage transformer. The NTP generator was placed in an especially developed contact chamber. The air flow was set at 3.0 L min^{-1}, brought inside from the pump, and touched the NTP reactor inside the chamber where the cold plasma is generated (cold plasma area). Here, the air is ionized and then enriched with oxidizing substances, comes out, and then bubbles in the nutrient solution (Figure 1); then, a large part of this air flow moves in the air of the HGS.

Since the conditions of cold plasma generation and air flow were fixed, the control of the quantity of reactive species was obtained by measuring the on/off time of the NTP generator. In particular, air flow through the NTP prototype was working for 10 min (6000 s) every hour to prevent root hypoxia, while the NTP operating time ranged from 0 to 360 s, according to the tested treatment.

2.2. Plant Growth Conditions

Four HGSs were placed inside separate growth chambers with a temperature of 24 ± 1 °C and relative humidity RH of 70 ± 5%. The photosynthetic photon flux density (PPFD) at the plant level was 150 µmol m^{-2} s^{-1}, with a 16 h photoperiod, and was generated by LED lamps (C-LED, Imola, Italy) with the following spectrum: 50% red radiation (peak at 650 nm), 33% blue radiation (peak at 450 nm), 17% green radiation (peak at 540 nm). The

light spectrum was measured with a spectrometer (FLAME-T-XR1-ES S/N: FLMT07829, Ocean Insight, Maybachstrasse 11, Ostfildern, D-73760 Germany).

Lettuce (*Lactuca sativa* var. *acephala* Alef. cv. Salad Bowl, Gargini Sementi, Capannori (LU), Italy) seeds were sown in 120-hole polystyrene trays with rockwool plugs (Grodan Plug®, Grodan Rockwool B.V., Roermond, The Netherlands), and covered with a layer of vermiculite. Trays were placed in a growth chamber for germination at a constant temperature of 25 °C for 48 h, in the dark. After seed emergence, the trays were transferred to a greenhouse till transplanting into the HGS, which took place 10 days after sowing, when the seedlings had two true leaves.

The HGS consisted of a box of transparent plastic (33 × 56 × 55 cm height; surface area of 0.185 m^2), equipped with a floating system containing 20 L of nutrient solution that hosted 100 plants; crop density was 541 plants m^{-2}). Nutrient solutions were prepared using tap water and appropriate amounts of analytical-grade salts. The nutrient solution had electrical conductivity (EC) of 2.34 dS m^{-1}, pH of 5.5, and the following ion composition (mg L^{-1}): 140.4 NO_{3^-}, 21.0 $HP_2O_4{}^-$, 264.0 K^+, 180.8 Ca^{2+}, 48.7 Mg^{2+}, 39.1 Na^+, 1.23 Fe^{2+}, 0.19 Cu^{2+}, 0.85 Zn^{2+}, 0.55 Mn^{2+}.

The nutrient solution was intermittently (10 min each hour) aerated to avoid root hypoxia. The air flux was generated by an air pump and, before reaching the nutrient solution, it passed through the NTP generator, which could be turned on with an independent operating time. Crop evapotranspiration was compensated on a daily basis by refilling the hydroponic tank with appropriate volumes of distilled water. In consideration of the large volume of the nutrient solution and the short growing period, during each experiment, the hydroponic solution was never changed.

2.3. Experiments

Four experiments were conducted between October 2019 and March 2020.

The aim of the first experiment was to define the maximal operation time of the NTP system that allowed for keeping the ozone concentration in the HGS (as determined using portable gas analyzer by Horiba, model APOA-370, HORIBA Ltd., Kyoto, Japan) as much as possible close to but not higher than the safe concentration for lettuce, which is 50 ppb [25]. This experiment was conducted without lettuce plants in the HGS, and the following operating times were tested: 0, 30, 60, 90, 120, 180, 240, 300, and 360 s. Air O_3 concentration was monitored throughout the whole NTP operation and in the following 30 min.

In the second experiment, we investigated the variation in pH and electrical conductivity, (EC), and the concentration of selected ions (NO_{3^-}, K^+, Fe^{2+} and Na^+) changed in plant-free nutrient solutions treated with NTP for the maximal safe working time (120 s every hour) that had been found in Experiment 1. In this experiment, nutrient solutions were sampled until 96 h 2, 4, 6, 8, 24, 72, and 96 h after NTP treatment.

In the third experiment (calibration), the effects of NTP on plant growth and leaf quality were investigated in lettuce plants grown in HGS for nine days. The nutrient solution was treated with NTP for 0 (control), 30, 60, and 120 s every hour.

In the fourth experiment (validation), the effects of NTP were investigated in lettuce plants grown for nine days in a nutrient solution treated with an NTP generator operating for 0 or 120 s every hour.

At the end of the experiment, three or four plants were sampled for the determination of leaf fresh (FW) and dry (DW) weight, and the content of nitrate, chlorophyll, carotenoid, and phenolic content, and antioxidant capacity. Chlorophyll *a* fluorescence was also measured on intact plants on the day before harvest.

2.4. Experiments Determining NTP Effects on Lettuce Plants

On the basis of the preliminary results, two independent experiments (Experiments 3 and 4) were conducted to evaluate the effects of NTP applied in the nutrient solution on the growth and crop quality of hydroponically grown baby leaf lettuce. In experiment 3 (a calibration) 0, 30, 60 and 120 s of NTP system operating times every hour were tested.

After nine days, plant samples were collected to measure shoot and root fresh FW and DW, dry matter percentage, and leaf number and area. The experiment was repeated twice with similar results.

In the calibration experiment, the maximal effectiveness operating time of NTP generator in the HGS was 120 s, so a validation experiment (experiment 4) was performed testing only two NTP system operating times with two replicates for each treatment: 0 (control) and 120 s. At the end of the experiment, leaf FW and DW, and the content of nitrate, total chlorophylls, carotenoids, and phenolics, antioxidant capacity, and chlorophyll *a* fluorescence were determined.

2.5. Determinations and Measurements

2.5.1. Ion Concentration of Hydroponic Nutrient Solutions

The concentrations of calcium, magnesium, sodium, iron, manganese, copper, and zinc in the nutrient solution were measured using an atomic absorption spectrometer (Varian Model Spectra AA240 FS, Agilent Technologies Australia [M] Pty Ltd., Mulgrave, Australia). A spectrophotometer (Perkin-Elmer UV/VIS Lambda 1; Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) was used for determining the concentration of phosphorus ($P–PO_4$) using the molybdenum blue method [26], and nitrate nitrogen ($N–NO_3$) using the salicylic–sulfuric acid method [27]. All analyses were performed in duplicate.

2.5.2. Crop Evapotranspiration and Growth

Crop evapotranspiration was assessed by measuring the volumes of water used to refill the hydroponic tank in each HGS.

The following growth parameters were assessed: fresh weight (FW) and dry weight (DW) of leaves and roots, root length (cm), number of leaves longer than 2 cm, and total leaf area (cm^2). DW was quantified after drying samples at 70 °C till constant weight. Leaf area was determined using a leaf area meter (MK2, Delta-T Devices, Cambridge, UK).

2.5.3. Leaf Content of Nitrates, Pigments, and Total Phenolics, and Antioxidant Capacity

Leaf $N–NO_3$ content was spectrophotometrically determined in dry samples (ground in a laboratory mill to a powder) extracted with distilled water (100 mg DW in 20 mL) at room temperature (20–22 °C) for 2 h, using the salicylic–sulfuric acid method [27].

Fresh leaf samples consisting of 12 mm diameter disks taken from distinct leaves were weighed (approximately 0.5 g FW) and soaked in 5 mL methanol in 10 mL test tubes. The tubes were sonicated four times for 15 min on ice and stored overnight at −20 °C. After separation of the supernatant, the extraction of the disks was repeated with 5 mL fresh methanol. The two supernatant aliquots were pooled, and, after proper dilution (1:10) with methanol, the absorbance of the extracts was read at 665.2, 652.4, and 470 nm. The concentrations of pigments chlorophyll *a*, chlorophyll *b*, and carotenoids were calculated according to Lichtentahler and Buschmann (2001) [28].

Total phenol content was determined using the Folin–Ciocalteu reagent [29]. Approximately 0.5 g of fresh leaf disks were homogenized in a mortar with 5 mL of 70% methanol (*v*/*v*) and extracted overnight at 4 °C in the dark under continuous agitation. After centrifugation (5 min, 10,000 rpm) at room temperature, the clear supernatant was collected and used for the subsequent analyses of total phenolic content and antioxidant capacity.

For phenolic content, 125 µL of leaf methanolic extracts was diluted in distilled water (1:4), mixed with the Folin–Ciocalteau reagent [29] to a final volume of 750 µL and vortexed. After 6 min, 1.25 mL 7% (*w*/*v*) Na_2CO_3 solution and 1 mL distilled water were added to each sample in a test tube incubated at room temperature in the dark for 90 min. Spectrophotometric readings were carried out at 765 nm, and results are expressed in terms of gallic acid equivalents per g FW (mg GAE g^{-1} FW) on the basis of a standard calibration curve.

Antioxidant capacity was measured in methanol extract samples using the FRAP assay [30]. The methanol extract was added to the FRAP reagent (1 mol m^{-3} 2,4,6-tripyridyl-

2-triazine TPTZ and 2 mol m^{-3} ferric chloride in 250 mol m^{-3} sodium acetate). and analyzed by spectrophotometry. Briefly, 0.1 mL of the methanol extract was added to 0.9 mL of the FRAP reagent, mixed, and kept at 20 °C for 4 min. Antioxidant capacity was calculated using a calibration curve prepared with standard solutions containing ferrous ion (Fe(II); 0–1000 µM) obtained from ferrous ammonium sulphate; results are expressed as µmol of Fe(II) g^{-1} FW.

2.5.4. Chlorophyll *a* Fluorescence

Chlorophyll *a* fluorescence was measured nine days after transplant using a portable fluorimeter (Handy PEA Hansatech Instruments Ltd. Narborough Road, King's Lynn, UK). Measurements were taken on leaves already after developing after the 2nd–4th day of treatment that entirely filled the area of the sensor (3rd–5th true leaves). Leaves were selected for the measurements and, after adaptation in the dark for 30 min using leaf clips, were exposed to an excitation light intensity of 3000 µmol m^{-2} s^{-1} emitted by three diodes. Chlorophyll *a* transient fluorescence obtained from the dark-adapted sample was analysed with the OJIP test [31], and phenomenological and specific indices were calculated.

2.5.5. Statistical Analysis

Each experiment was conducted twice with similar results, and data from a representative run are reported here. One- or two-way analysis of variance (ANOVA) was performed to determine the significance of the effects of time and/or NTP treatment and their interaction on measured quantity. Mean values were separated by the LSD test ($p < 0.05$). Statistical analysis was performed using Statgraphics Plus 5.1 (Manugistic, Rockville, MD, USA) and GraphPad (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Ozone Concentration in HGS Produced by NTP Generator with Different Operating Times

The effects of different operating times of the NTP system used to treat the nutrient solution on the O_3 concentration in the air of HGS were tested without plants in the hydroponic system (experiment 1).

There was a positive linear relationship between maximal O_3 concentration in the air inside the HGS and NTP operating time in the range from 30 to 360 s (Figure 2): at the lowest (30 s) and highest (360 s) tested NTP doses, O_3 concentration peak was 18 and 151 ppb, respectively. In all treatments, O_3 concentration decreased to about 5 ppb within 30 min (Figure 3).

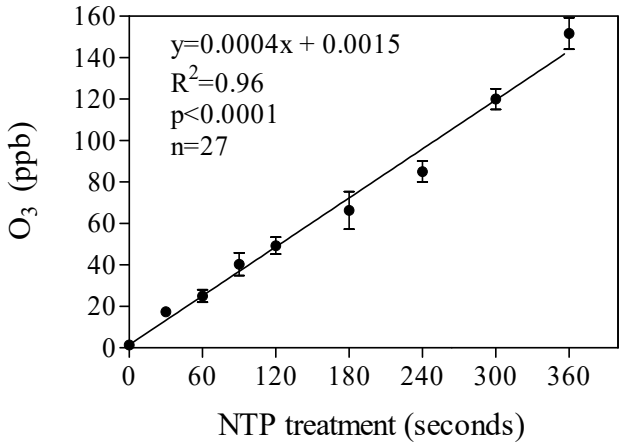

Figure 2. Linear regression between peak ozone (O_3) concentration in air inside hydroponic growing system and operating time of NTP generator used to treat nutrient solution in hydroponic tank. Each value is mean (±SE of three measurements taken in three different occasions over 24 h of system operation (experiment 1). Regression equation and determination coefficient (R^2) shown inside graph.

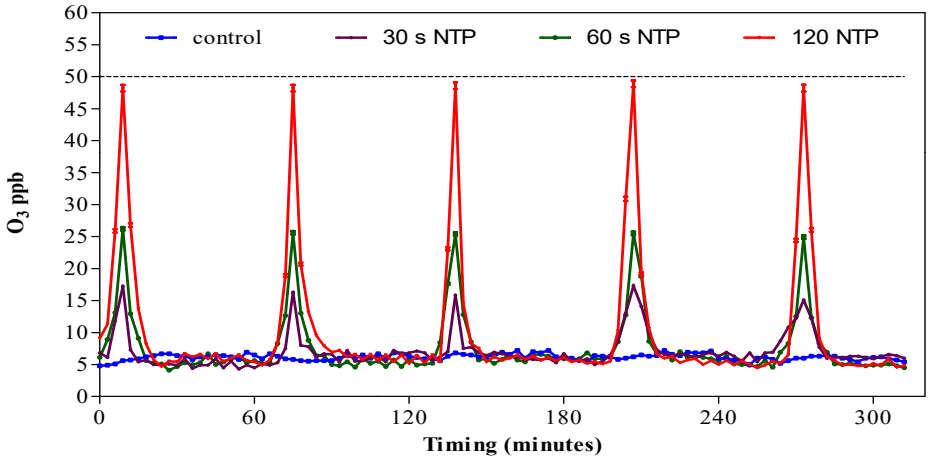

Figure 3. Time course (0–300 min) of ozone (O_3) concentration in air inside hydroponic growing system with nutrient solutions treated with NTP for 0 (control), 30, 60, or 120 s every hour, without plants grown in hydroponic tank (experiment 1). Each value is mean (±SE) of measurement taken in three different occasions over one day of system operation (Experiment 1). O_3 measurements were recorded every 3 min.

Lettuce is an ozone-sensitive species, and the O_3 stress threshold reported in the literature for this species is 50 ppb [25], corresponding with a longer NTP operating time than 120 s. Therefore, NTP treatment should not exceed 120 s every hour to avoid O_3 injuries to the plants.

3.2. Effects of NTP on Ion Concentrations of Hydroponic Nutrient Solution

The effects of NTP treatment on the ion concentration of hydroponic solution were investigated without plants in the floating tank (experiment 2). After 96 h, N–NO_3 concentration increased (+4%) in the NTP-treated nutrient solution compared to the control, whereas the levels of K, Na, Fe (Figure 4), and Zn (Figure S1) decreased by 2.7%, 9.0%, 22.6%, and 5.9%, respectively. In both the control and the NTP-treated nutrient solutions, the concentrations of P–PO_4, Ca, and Mg diminished with time, while no significant variations were observed for EC and the content of Cu and Mn concentration (Figure S1).

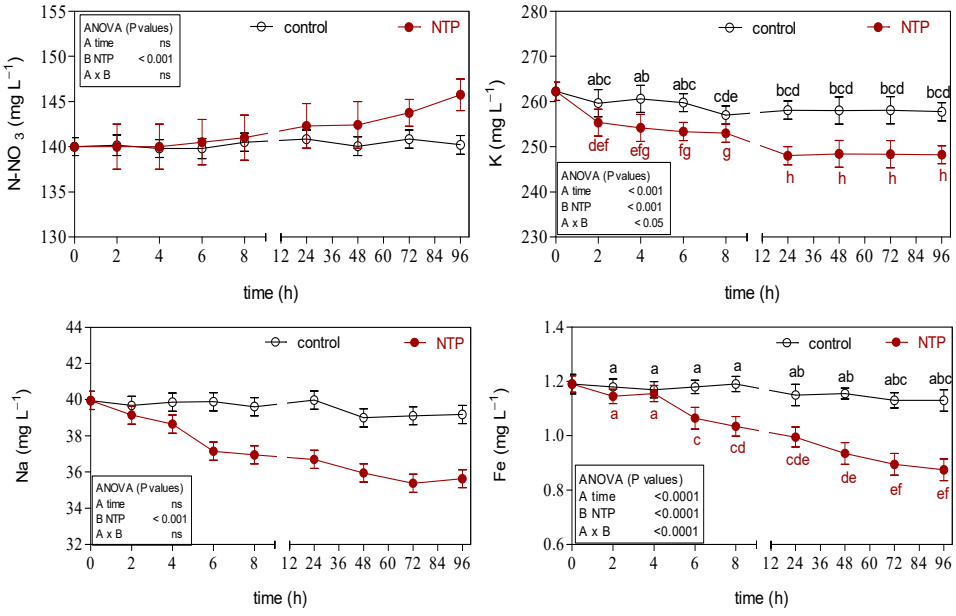

Figure 4. Effect of NTP treatment (120 s every hour) of nutrient solution without plants on concentration of nitrate (N–NO_3), potassium (K), sodium (Na), and iron (Fe) (experiment 2). Each value is mean of

two replicates (±SE). ANOVA results shown inside graphs. Different letters indicate significant differences between control and NTP treatment according to LDS test at 5% level. Different letters indicate significance at $p < 0.05$ and 0.01, respectively.

At the end of the experiment, some mineral precipitation was observed in the closed hydroponic solution, and, using a scanning electron microscope (SEM-EDX), calcium, magnesium and potassium phosphate minerals, and iron were identified as main components (data not shown).

3.3. Effect of NTP on Lettuce Leaf Production and Quality

In experiment 3, the nutrient solution in the hydroponic tank hosting lettuce plants was treated for 0 (control), 30, 60 and 120 s every hour. NTP did not affect the pH and EC of the nutrient solution, which oscillated between 5.59 and 6.55, and between 2.38 and 1.77 dS m^{-1}, respectively (Figure S2). Total water uptake was similar in both treatments and averaged 6.66 ± 0.08 L for HGS (approximately to 36 L m^{-2}).

The production of fresh biomass and leaf area tended to increase with the duration of NTP treatment (Table 1). When NTP treatment lasted 120 s, shoot and root FW, leaf number and area, and root length increased by 16.4%, 18.2%, 40.1%, 10.1%, and 54.1%, respectively, with respect to the control. Significant differences between controls and NTP-treated plants for these growth parameters were also observed when NTP had been applied for 60 s (Table 1). No significant effect of NTP was found in shoot and root DW (data not shown) while a significant reduction in DW/FW percentage ratio was observed in the 60 and 120 s NTP treatments with respect to the control (Table 1).

Table 1. Effects of NTP treatment (0, 30, 60, or 120 s every hour) of nutrient solution on shoot and root fresh weight (FW), leaf number, leaf area, root length, and DW/FW percentage ratio in both leaves and roots of baby leaf lettuce plants grown in an indoor hydroponic system for nine days (Experiment 3).

Treatments	Shoot FW (g Plant^{-1})	Root FW (g Plant^{-1})	Leaf Number (Plant^{-1})	Leaf Area (cm^2 Plant^{-1})	Root Length (cm)	Leaf DW/FW (%)	Root DW/FW (% FW)
Control	3.10 ± 0.07 [Z] b	0.33 ± 0.00 b	8.33 ± 0.47 c	46.80 ± 0.92 b	8.94 ± 0.28 c	3.34 ± 0.07 a	2.47 ± 0.26 a
NTP–30 s	3.06 ± 0.04 b	0.34 ± 0.02 b	9.67 ± 0.47 bc	47.04 ± 0.09 b	11.83 ± 0.36 b	3.79 ± 0.23 a	2.39 ± 0.25 a
NTP–60 s	3.55 ± 0.10 a	0.34 ± 0.03 b	11.00 ± 0.82 ab	48.38 ± 0.43 b	11.17 ± 0.0.36 b	2.40 ± 0.23 b	1.53 ± 0.28 b
NTP–120 s	3.61 ± 0.06 a	0.39 ± 0.02 a	11.67 ± 1.25 a	51.53 ± 0.31 a	13.78 ± 1.10 a	2.54 ± 0.34 b	2.05 ± 0.56 ab
Significance [y]	**	*	**	*	***	**	NS

[Z] Each value is mean of three replicates (±SE). [y] Data analyzed by one-way ANOVA, and for each column, different letters indicate significant differences between treatments according to LDS test at 5% level. NS, *, **, ***, are not significant, significant at $p < 0.05$, 0.01, 0.001, respectively.

All NTP treatments increased the leaf content of total carotenoids and chlorophyll contents with respect to the control, while antioxidant capacity and total phenol content significantly increased only in the 60 and 120 s NTP treatments (Figure 5 and Table S1. No significant differences were observed among treatments as regards leaf NO_3 content, which was much higher at harvest (2440 ± 45 mg Kg FW^{-1}) than that at transplanting (1037 ± 33 mg Kg FW^{-1}).

In experiment 4, the nutrient solution was treated with NTP for 120 s every hour. After nine days, no difference in the water consumption was observed between control, and h and standard deviation for the control and NTP treatment were 27.6 ± 1.9 and 26.8 ± 2.70 L m^{-2}, respectively.

In the control and NTP-treated hydroponic solutions, the concentration of all nutrients Na, and EC declined during the experimental period, while pH increased (Figures 6 and S2). At the end of the experimental period, no significant differences were found between the two nutrient solutions, with the exception of Fe and Zn (Figures 6 and S2) that decreased to a greater extent in the 120 s NTP treatment than that in the control.

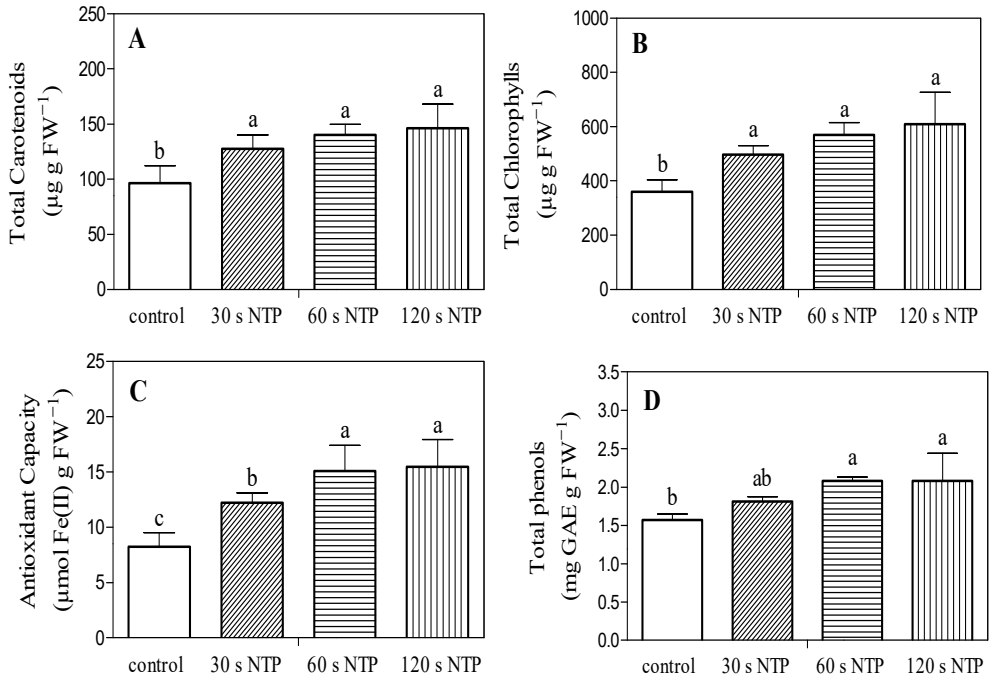

Figure 5. Effect of NTP treatment (0, 30, 60 or 120 s every hour) of nutrient solution on leaf content of carotenoids (**A**), chlorophylls (**B**), antioxidant capacity (**C**), and total phenols (**D**) in lettuce plants grown in indoor hydroponic system for nine days. Each value is the mean of three replicates (±SE). Different letters indicate significant differences between treatments according to LDS test at 5% level.

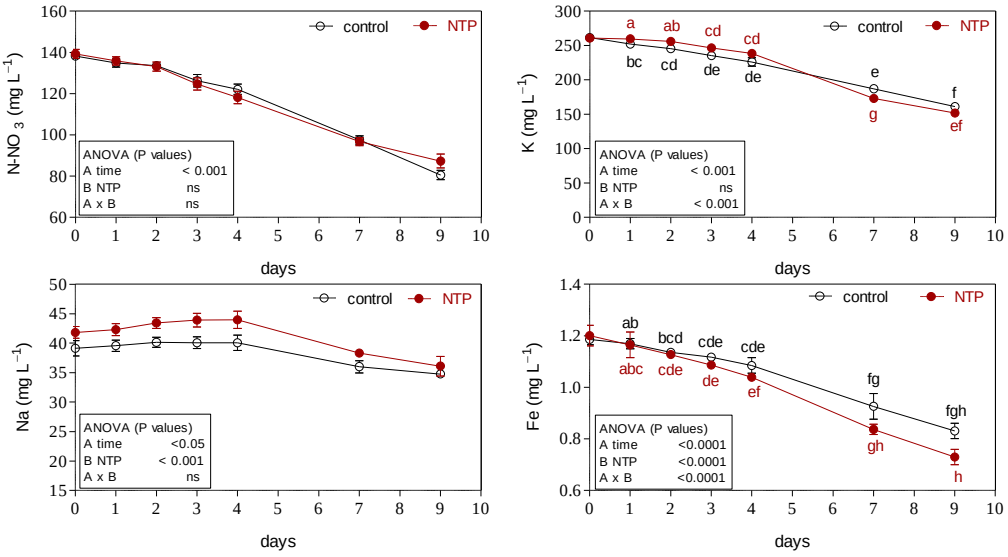

Figure 6. Effect of NTP treatment (120 s every hour) on concentration of nitrate (N–NO_3), potassium (K), calcium (Ca), sodium (Na) and iron (Fe) of hydroponic nutrient solution with plants (Experiment 4). Each value is the mean of two replicates (±SE). ANOVA results shown inside graphs. Different letters indicate significant differences between control and NTP treatment according to LDS test at 5% level. Different letters indicate significance at $p < 0.05$ and 0.01, respectively.

The NTP treatment stimulated plant growth and after nine days shoot and root FW, leaf number and area, and root length were 12.3%, 15.0%, 27.8, 20.3 and 24.6% greater, respectively, in NTP-treated plants than those in the controls (Table 2). No significant differences were found in dry matter content of lettuce plants with NTP treatment (Table 2).

Table 2. Effect of NTP treatment (120 s every hour) of nutrient solution on leaf and root fresh weight (FW), leaf number and area, (FW), leaf number, leaf area, root length, and DW/FW percentage ratio in leaves and roots of baby leaf lettuce plants grown in an indoor hydroponic system for nine days (Experiment 4).

Treatments	Shoot FW (g Plant^{-1})	Roots FW (g Plant^{-1})	Leaf Number (n Plant $^{-1}$)	Leaf Area (cm^2 Plant^{-1})	Root Length (cm)	Leaf DW/FW (%)	Root DW/FW (% FW)
Control	3.99 ± 0.09 [Z] b	0.40 ± 0.1 b	9.0 ± 0.3 b	61.17 ± 6.28 b	13.00 ± 0.11 b	3.28 ± 0.10 a	3.03 ± 0.04 a
NTP–12 s	4.48 ± 0.03 a	0.46 ± 0.1 a	11.5 ± 0.5 a	73.59 ± 3.35 a	16.20 ± 0.50 a	2.87 ± 0.14 a	2.92 ± 0.26 a
Significance [y]	*	*	*	*	**	NS	NS

[Z] Each value is the mean of two replicates (±SE). [y] Data were analyzed by one-way ANOVA, and for each column, different letters indicate statistically significant differences according to LDS test at 5% level. NS, *, ** are not significant, significant at $p < 0.05$, 0.01, respectively.

The NTP treatment significantly affected leaf quality parameters (Table 3). The leaf concentration of carotenoids, chlorophylls, and total phenols, and the antioxidant capacity higher in the NTP-treated plants (by 37.1%, 22.0%, 40.2% and 36.9%, respectively) than those in the controls. Leaf nitrate content was not affected by NTP treatment (data not shown) as observed in the previous experiment.

Table 3. Effect of NTP treatment (120 s every hour) of the nutrient solution on leaf content of total carotenoids, chlorophylls content, antioxidant capacity and total phenols content of baby leaf lettuce plants grown in hydroponic growing system (HGS) for nine days.

Treatments	Total Carotenoids (μg gFW^{-1})	Total Chlorophylls (μg gFW^{-1})	Antioxidant Capacity (μmol Fe(II)g FW^{-1})	Total Phenols (mg GAE gFW^{-1})
Control	94.60 ± 10.18 [Z] b	485.99 ± 34.28 b	14.23 ± 0.46 b	2.68 ± 0.05 b
NTP-120 s	135.70 ± 10.49 a	616.01 ± 10.13 a	19.95 ± 0.25 a	3.67 ± 0.09 a
Significance [y]	*	*	**	**

[Z] Each value is mean of two replicates (±SE). [y] Data analyzed with one-way ANOVA, and in each column, different letters indicate significant difference between treatments according to LDS test at 5% level. *, ** significant at $p < 0.05$ and 0.01, respectively.

3.4. Chlorophyll a Fluorescence

The effect of NTP supply in the nutrient solution on the photosynthetic system of lettuce leaves was investigated by measuring the fast chlorophyll *a* fluorescence transient at harvest. As presented in Figure 7 (left) NTP affects two fluorescence parameters related to PSI activity: the ϕRo (ϕR0 = δRo*ϕPo*ΨEo; Quantum yield for the reduction in end acceptors of PSI per photon absorbed); δRo (δR0 = (1 − Vi) (1 − Vj), the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors).

The two fluorescence parameters increased significantly in NTP-treated plants. Among chlorophyll *a* fluorescence parameters obtained from OJIP transients, the amplitude of the IP phase ($\Delta V_{IP} = 1 - V_I = (F_M - F_{30\ ms})/(F_M - F_0)$; efficiency of electron transport around the PSI to reduce the final acceptors of the electron transport chain, i.e., ferredoxin and NADP) that thus reflects the relative PSI content [32], was higher in NTP-treated plants than that in nontreated plants (Figure S1). Parameters tFM and Sm/tFM were significantly affected by NTP treatment (Figure 7 graphs on the right). The time to reach maximal fluorescence (Tfm), which is an indicator of QA reduction rate of the PSII acceptor, significantly decreased in NTP treated plants. Additionally, Sm/tFM (average fraction of open reaction centers during the time needed to complete their closure) was substantially increased in NTP-treated lettuce plants.

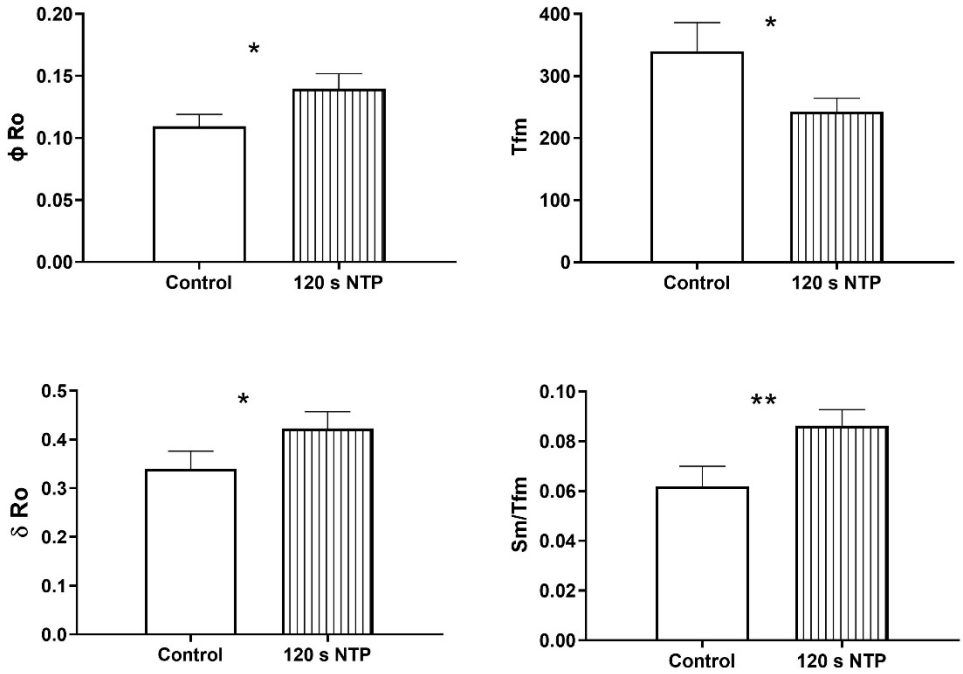

Figure 7. Effects of NTP treatment (120 s every hour) of nutrient solution on chlorophyll *a* fluorescence of leaves of lettuce plants grown hydroponically with nutrient solution treated with NTP (120 s). (left) Parameters related to PSI activity, the ϕRo (quantum yield for reduction in end acceptors of PSI per photon absorbed) and δRo (efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors). (right) Tfm: time to reach maximal fluorescence and Sm/tFM: average fraction of open reaction centers during time needed to complete their closure. Each value is mean of two replicates (±SE, n = 7). Pairwise comparisons between means performed using a two-tailed unpaired Student's *t* tests assuming equal variances (* $p < 0.05$; ** $p < 0.01$).

4. Discussion

4.1. Effects of NTP Treatment on Hydroponic Nutrient Solution

Water quality and mineral composition of a nutrient solution play a crucial role in plant growth and development. To understand the effects of NTP on mineral nutrition we investigated the direct effect of this treatment on the ion composition of the nutrient solution in experiments without plants. In the nutrient solutions treated with NTP, the level of N–NO_3 increased while an opposite result was found for Na, Fe, and Zn. The NTP also contributed to stabilizing the pH of air-bubbled nutrient solution, since in the control, pH increased with time (Figures 4 and S1).

Radicals generated in plasma typically include ozone (O_3), NO, and OH radicals [33]. The species (OH, O and NO radicals) formed with the NTP discharge at the gas–liquid interface and their products (NO_{3^-}, NO_{2^-}, H_2O_2) contribute to the effects on the chemical characteristics of the water, such as a decrease in pH and increase in conductivity and redox potential [34].

Ozone microbubbles as a disinfecting technology for hydroponic solutions significantly change the ion concentration (N–NO_3, N–NH_4, PO_4, K, and Ca) of nutrient solutions in various hydroponically grown plant species [33,34]. In this study with lettuce grown in a floating system, a slight decrease in Fe and Zn ion concentration was observed in the NTP treatment.

Plasma systems are used to enrich water or media with chemical compounds produced by the plasma for fertilization and sanitation [9,35]. For instance, after 30 min plasma treatment, the water NO_3 concentration increased from 0 to 16.22 mg L^{-1} [9].

Plasma treatment of water results in significant changes in various properties such as pH, oxidation-reduction potential (ORP), electrical conductivity, and concentration of ROS

and RNS [1]. However, the composition of plasma-generated RONS depends on the feeder gas (source), plasma device setting and environmental conditions.

Lo Porto et al. [36] investigated the effects of plasma-activated water on seed germination and growth using longer treatment (1 and 5 min) than those applied in the present study. The authors found that plasma treatment significantly reduced water pH and led to the formation of RONS. The concentration of NO_3^-, NO_2^- and H_2O_2 were dose-dependent; longer treatment time resulted in higher concentrations of NO_3^- and H_2O_2. Moreover, PAW induced positive effects on germination rate and subsequent plant growth [36]. Similarly, in our study, there was an increase in NO_3^- level in NTP-treated nutrient solution, while the pH of water was not affected by NTP treatment probably due to the adjustment of pH before irrigation [13] and to the presence of bicarbonate in the irrigation water (2.5 mM), which neutralized the H^+ protons produced by the NTP generator.

4.2. Effects of NTP Treatment on Plant Growth

In experiments 3 and 4, leaf, root, and total biomass, leaf number and area, and root length increased in NTP-treated plants compared with the controls (Tables 1 and 2, Figure 8). However, dry matter accumulation was not significantly affected by NTP. These findings agree with those reported by other authors [11,14,37]. Safari et al. [14] observed that 1 min plasma treatment positively affected the shoot and root lengths as well as leaf area in *Capsicum annuum*. Moreover, the NTP effects were dependent on the exposure time, leading to reduced growth after 2 min NTP treatment.

Figure 8. Baby leaf lettuce plants grown in indoor hydroponic growing system (HGS) for nine days after transplant in the control nutrient solution (**on the left**) or in a nutrient solution treated with NTP for 120 s every hour (**on the right**).

Similarly, longer roots were observed in soybean [38], green bell pepper [39], Arabidopsis [37], and tomato [11]. In the same way an increase in leaf area was observed in *Arabidopsis thaliana* [40], in tomatoes [9,41,42], and peppers [9]. The growth and yield

of spinach, tomato, and pepper plants increased when these species were irrigated with NTP-treated water [1]; the authors ascribed this effect to the increased levels of NO_{3^-} and NTP-induced acids, metastable peroxides, and ions in the treated water.

Recent evidence [32] suggests that the RNS and ROS produced by plasma treatment are responsible for the increased growth and fecundity of *Arabidopsis thaliana* plants. ROS and RNS activate plant defense and stimulate growth. For example, nitric oxide (NO), which is one of the reactive nitrogen species generated by plasma, is a key signaling molecule in plant growth, development, and response to stress [43,44].

Both ROS and RNS in plants can play a dual positive and negative role depending on the amount produced [37], and the duration of the plasma treatment is crucial.

In the present study, no significant effects of NTP treatment were found in plant water and mineral uptake, as no important differences in the composition of the nutrient solution of treated or not treated with NTP were observed between the controls and the NTP-treated plants after nine days of treatment (Figure 6). However, NTP-treated plants produced greater fresh biomass, suggesting an increase in water use efficiency. For example, in Experiment 2, WUE was 2.46 g DW L^{-1} in the control plants, and 2.62 g DW L^{-1} in those grown in nutrient solution treated with NTP. In a recent study conducted on two cultivars of Salanova lettuce with green or red leaves, crop WUE was increased by the NTP treatment of the nutrient solution as compared to the control plants [45]. Similar results were found in *Arabidopsis thaliana* [40].

In our study, total chlorophyll, total carotenoid, and total phenol content, and antioxidant capacity increased in NTP-treated plants, and this trend was noted in both independent experiments (Figure 5, Tables 3 and S1).

An increase in total chlorophyll content was reported in the leaves of soybean treated with plasma [38]. Babajani et al. [17] reported that chlorophyll and carotenoid content increased to 39% and 32% for plasma-treated seedlings, respectively, then that for the control in *Melissa officinalis* treated with zinc oxide or selenium nanoparticles.

The highest values of antioxidant capacity and total phenol contents detected in the NTP-treated plants may be related to the reaction of plants against the stress effects of NTP.

The plant response to stressors promotes the synthesis of phenolic compounds with antioxidant function to defend against oxidative damage and this could be improving the nutraceutical quality of vegetables [46]. For example, in lettuce plant an increase in total phenols content was observed to counteract the generation of ROS under stressful light intensity [38].

Recently, Adhikari et al. [47] reviewed the role of NTP as an efficient and environmentally safe tool for inactivating phytopathogenic microorganisms. As plasma produces ROS and RNS, which are signaling molecules regulating disease stresses in plants, the induction of disease tolerance or resistance can be triggered by plasma treatment. In another study on tomatoes [41], NTP-treated water irrigation enhanced endogenous H_2O_2 and NO levels in tomato seedlings, which in turn modulated the synthesis of several phytohormones ethylene, salicylic acid, and jasmonic acid) in plant cells [41]; moreover, RONS present in plasma-treated water influence the endogenous RONS level. Salicylic acid promotes the synthesis of flavonoids and other phenolic compounds, and both salicylic and jasmonic acids regulate plant vegetative growth, seed germination, root growth, nutrient uptake, water relations, ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity, chlorophyll content and activities of antioxidant enzymes. Therefore, it is possible that NTP activates upstream via RONS, various plant hormone signaling pathways that synergistically promote the biosynthesis of secondary metabolites.

There were no observable effects of NTP supply in the hydroponic nutrient solution on the leaf NO_3^- content ($p > 0.05$), which is an important quality parameter in the lettuce [48], as the amount of this ion generated by the NTP treatment was negligible (+2% with respect to the untreated nutrient solution).

Chlorophyll *a* Fluorescence

Chlorophyll fluorescence was determined for evaluating the effects of NTP treatments on plant photosynthetic performances.

The higher δRo and ϕRo reported in NTP-treated plants indicate a more dynamic electron transfer between the two photosystems and towards end electron acceptors of PSI [49,50] thus suggesting that NTP treatment might affect electron flow on the PSI acceptor side.

The IP phase is connected to the relative abundance of PSI as compared with PSII [51], and previous experiments reported the IP phase as the most closely related region of fluorescence transient to Pn [52,53]. Thus, the higher IP phase reported in NTP-treated plants might be connected to the increase in root length and LAI observed in these plants. Moreover, the amplitude of the IP phase, was found to increase in sun leaves than in shade leaves [32], during the noontime hours [52] and to counterbalance the effects of abiotic or biotic stress [49], which agree with the higher PSI/PSII ratio. For example, in ozone-damaged poplar plants, the remaining foliage (young leaves in the upper section of the crown) showed an increase in IP phase and net photosynthesis, suggesting that high PSI/PSII ratio allows for a quick reduction in ferredoxin and consequently greater efficiency in carbon reduction [49].

A reduction in time to reach Fm (Tfm) by NTP treatment enhanced the average redox state of QA in the time span from 0 to Tfm (Sm/Tfm). Generally, Tfm is higher when electron transport is blocked somewhere [50]; thus, our results suggest that the rate of light trapping and electron transport was enhanced, indicating that changes induced by NTP treatment increased the average redox state of QA. Previous studies on mung bean reported that the time span from 0 to Tfm (Sm/Tfm) was reduced by foliar application of salicylic acid (SA), and this condition was positively correlated to plant tolerance of salt stress [54]. On the other hand, Chen and Cheng [55] reported a closure of PSII RCs in chlorotic leaves, suggesting the acceptor side of PSII was severely damaged, most likely due to the over-reduction in PSII as indicated by decreased Sm/tFm [55].

Our data showed that Sm/Tfm was higher in NTP-treated leaves than in normal ones, suggesting that the increased average fraction of open RCs (Sm/tFm) in NTP leaves might reflect an enhancement of photosynthetic electron transport rate.

Overall, these results indicate that the photosynthetic electron transport chain up to the reduction in end acceptors of PSI is improved in the leaves of hydroponically grown plants in nutrient solution treated with NTP. NTP treatment also induces a photosynthetic adjustment in the leaves, which leads to the improved efficiency to reduce the final acceptors beyond the PSI. Current findings thus suggest evidence for a positive stimulatory effect of ozone on photosynthetic performance leading to longer roots, larger leaf area, root length, and LAI) and the accumulation of bioactive compounds. Recent studies proposed that low concentrations of stressor agents (such as ozone) induce hormetic effects through the stimulation of photosynthesis as well the activation of plant stress defense mechanisms [56,57]. In this context, the phenomenon of hormesis in plants can be used as a strategy to improve the quality production of crops [58]. Nevertheless, in our work, the high efficiency of the photosynthetic apparatus induced by NTP treatment did not increase dry matter accumulation, probably due to the short growth cycle. In some of the literature, dry matter production increased in NTP-treated plants, but did not change or even decreased in other works [13].

5. Conclusions

This work demonstrated that short (1–2 min) NTP treatment applied to hydroponic nutrient solution enhanced leaf fresh biomass production as a consequence of a eustress response that also stimulated the secondary metabolites. Our results are consistent with previous findings on the positive effects of NTP on plant growth, and suggest a possible application of the NTP technology in hydroponic cultures.

Further work is necessary to assess the plant response to NTP in longer cultivation in greenhouse hydroponic systems.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8030251/s1. Figure S1: effect of NTP treatment (120 s every hour) on concentration of calcium (Ca), magnesium (Mg), phosphate (P–PO_4), zinc (Zn), pH and electrical conductivity (EC) of nutrient solution without plants (Experiment 2). Data refers to treatment of 120 s each hour of NTP supply in the nutrient solution. ANOVA results shown inside graphs. Different letters indicate significant differences between control and the NTP treatment according to LDS test at 5% level. Figure S2: effect of NTP treatment (120 s every hour) on concentration of calcium (Ca), magnesium (Mg), phosphate (P–PO_4), zinc (Zn), pH and electrical conductivity (EC) in nutrient solution with plants (Experiment 3). ANOVA results shown inside graphs. Figure S3: relative amplitude of I–P phase ($V_{IP} = 1 - V_I = (F_M - F_{30ms})/(F_M - F_o)$, in control and NT-treated plants. Data reported as mean ± standard error (n = 7). Pairwise comparisons between means performed using a two-tailed unpaired Student's *t* tests assuming equal variances (* $p < 0.05$); Table S1: effect NTP supply (obtained by changing operating time from 0 to 120 s) on total carotenoid content, chlorophyll content, antioxidant capacity, and total phenol content dry matter (DW) based on baby leaf lettuce grown in an indoor floating system for nine days. Data were analyzed by one-way ANOVA, and for each column, different letters indicate statistically significant differences according to LDS test at 5% level. *** are significant at $p < 0.001$ respectively.

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