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Effect of Ozonated Fertigation in Pepper Cultivation under Greenhouse Conditions

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Abstract: Ozone has a high oxidizing power avoiding the presence of residues, so it is a good candidate for use in organic farming. However, its application in fertigation has been little studied. Two experiments were conducted simultaneously to check the aims of this work. The aim of the first experiment was to study pepper response under the nutrient solution supply and the application of O_3 and its interactions, and the aim of the second experiment was to determine the optimal dosage of O_3 to be supplied, from the point of view of plant development and nutritional and physiological status in both experiments. We conclude that O_3 supply via fertigation increased pepper biomass production. According to the results obtained, we recommend that the optimal dosage of O_3 in pepper plants be from 0.18 to 0.36 mg L^{-1} due to its higher associated biomass production. Under these dosages, pepper plants showed a higher leaf area and higher photosynthetic pigment concentration. Similarly, under these dosages, N, P, and K plant uptake were higher, allowing a higher synthesis of bioassimilates.

Keywords: biomass; chlorophyll; nitrogen; phosphorus; potassium; proline; sugars

1. Introduction

The current demand for quality horticultural products considering the international standards of safety, good agricultural practices (GAP), and sustainable development has led to the development of practices such as organic fertilization, the rational use of water and nutrients, and the biological control of pests and diseases, among others. With respect to the rational use of water and nutrients, different technologies have been studied such as sub-irrigated planter systems [1], recirculation in hydroponic systems [2], and the implementation of cascade cropping systems [3,4]. Nevertheless, the reuse of the leachate increases the risk of pathogens such as *Verticillium dahliae* and *Phytophthora capsici* [5], which are disseminated by water [6].

Disinfection strategies for soils, substrates, and irrigation water include the use of different chemical compounds such as methyl bromide, chloropicrin, dazomet, and metam sodium, which are currently banned in the European Union [7]. Nowadays, the agricultural implementation of sustainable alternatives for cleaning urban wastewater through oxidative degradation of organic matter are based on the application of other chemical compounds with lower toxicity, such as hydrogen peroxide (H_2O_2) [8], chlorine (Cl_2), peroxyacetic acid ($C_2H_4O_3$) [9,10], and ozone (O_3) [11], the most recently implemented.

Ozone has a higher oxidizing power than H_2O_2, Cl_2, or $C_2H_4O_3$. The molecule of O_3 is unstable; it decomposes into hydroxyl radicals (OH^-), hydroperoxide (HO_2^-), and superoxide (O_2^-), which are responsible for microbial inactivation through a process of the cytoplasmic membrane and cell-wall destruction [12]. These properties confer to this compound a high bactericidal, virucidal, and fungicidal effect. Therefore, O_3 is considered the fastest method for inactivation or elimination compared to other disinfecting agents

that require penetration of the cell membrane to be effective. Consequently, O_3 is also used in other fields such as industrial and medical processes, for instance, disinfection of COVID-19 material [13]. Nevertheless, it is necessary to highlight its toxicity. The European Commission established that the human exposure hazard to O_3 is 120 μg m^{-3} with a maximum daily exposure of 8 h (Directive 2008/50/CE) [14].

Regarding the effects of high concentrations of tropospheric O_3 on different crops, there are several references in the literature. For instance, Macdowall et al. [15] reported the presence of necrosis in tobacco leaves (weather fleck) in environments with up to 40 ppb of O_3 gas during 14 continuous hours at night. Likewise, Kumari et al. [16] reported a growth reduction in *Beta vulgaris* under a concentration of 70 ppb of O_3. Nevertheless, the experiments carried out with dissolved O_3 in irrigation water are scarce. For instance, Graham et al. [17], Graham et al. [18], and Martínez-Sánchez and Aguayo [19] reported the positive effect of the application of dissolved O_3 in irrigation water in five nursery crops, in tomato, and in pepper, respectively. Therefore, we aimed to check whether this positive effect occurs with dissolved O_3 in pepper crops in two different scenarios: under irrigation with water or fertigation with a standard nutrient solution, and also to determine the optimal concentration of O_3 supply by focusing on the changes caused at the physiological and nutritional level in pepper plants.

2. Materials and Methods

2.1. Location and Facilities

The experiments were conducted in a mono-tunnel greenhouse at the University of Almería (36°49′ N, 2°24′ W). The greenhouse was covered by polycarbonate stratified with transparent fiberglass together with a white mesh of 25% shading. Weather conditions inside the greenhouse were recorded with HOBO SHUTTLE sensors (model U12–13, Onset Computer Corp., Bourne, MA, USA). The average values of temperature, relative humidity, and photosynthetically active radiation (PAR) recorded during the experimental period were 27.05 ± 1.4 °C, 62. 89 ± 2.4%, and 16.62 ± 1.3 MJ m^{-2} day^{-1}, respectively. The cultivation was carried out on metal tables with a surface of 12 m^2 and a height of 80 cm.

2.2. Plant Material

Capsicum annum L. var. Claque F$_1$ (Rijk Zwaan) seedlings were purchased from a commercial nursery and then transplanted into 4 L plastic containers filled with blond peat moss. Under each pot, plastic buckets were used to collect generated leachate. The experiment lasted for 50 days (from 26 April to 15 July, 2019). The planting density was 2 plants m^{-2}.

2.3. Experimental Design and Treatments

Two experiments were conducted simultaneously with pepper plants. The first experiment was carried out to check whether there were differences in physiological and nutritional properties in pepper plants irrigated with water or nutrient solution with or without O_3. The experimental design was a bifactorial split-plot (fertigation or not and supplies of O_3 or not) completely randomized with four blocks and four plants (pots) per treatment combination block in each treatment. The second experiment was carried out to test the effect of increasing O_3 concentrations in the nutrient solution on pepper plants at the physiological and nutritional levels. The experimental design was composed of four levels of O_3 concentration, four blocks, and four plants (pots) per block (Table 1). The chemical composition of irrigation water and nutrient solution is shown in Table 2. The nutrient solution used was the same supplied by local growers of this crop and was achieved with irrigation water and added simple fertilizers.

Table 1. Characterization of fertigation treatments. Irrigation water (NS_0) and fertigation with the standard nutrient solution (NS_1); $O_{0, 0.18, 0.36, 0.54}$: ozone at different concentrations (0, 0.18, 0.36, and 0.54 mg L^{-1}, respectively).

	Fertigation	O_3 Supply
Experiment 1	NS_0	O_0
	NS_0	$O_{0.54}$
	NS_1	O_0
	NS_1	$O_{0.54}$
Experiment 2	NS_1	O_0
	NS_1	$O_{0.18}$
	NS_1	$O_{0.36}$
	NS_1	$O_{0.54}$

Table 2. Chemical characterization of the tap water and the standard nutrient solution (NS). Electrical conductivity (EC) and macronutrient concentration are expressed in dS m^{-1} and mmol L^{-1}, respectively.

	EC	pH	NO_3^-	$H_2PO_4^{2-}$	SO_4^{2-}	Cl^-	K^+	Ca^{2+}	Mg^{2+}	Na^+
Water	0.9	8.1	0	0	1.1	3.5	0	2	1.4	2.6
NS	1.3	6	6	0.7	1.1	3.5	3	2	1.4	2.6

To generate O_3 for its application in the fertigation system, the Evozon3 system (Evo Industria, Malaga, Spain) was used. This system is based on the capture of ambient air through a centrifugal pump (3 kg cm^{-2} of pressure and 3.5 m^3 h^{-1} of flow rate), where O_2 is filtered with zeolite and sent to a concentrator at a flow of 8 L min^{-1} to reduce the humidity to 14%. Then O_2 is ionized with a high voltage current of 9000 V, releasing one of the atoms of oxygen and causing the binding of this atom with another O_2 molecule, resulting in O_3 molecules. The O_3 produced with a concentration of 10 g h^{-1} is then injected by a submersible Venturi system and a flash reactor, which favors its mixing and homogeneity in a 120 L water tank from the network.

The equipment also has a pH and oxidation-reduction potential (mV) probe (PHTK-160 Digital PH ORP (2 in 1), Teckoplus Ltd., Trademart Dr, Kowloon Bay, Hong Kong). The system works for 30 min, reaching around 850 to 1000 mV, and after this period the ozonated water had the optimum conditions for its use in the experiments. There is a table conversion between power redox and O_3 concentration to check continuously that the optimal conditions of the experiment were being conducted, following the recommendations given by TopOzono [20] and the advice given by local growers (Table 3).

Table 3. Conversion between power redox (mV) and O_3 concentration (mg L^{-1}).

Power Redox (mV)	O_3 Concentration
0	0
225	0.18
450	0.36
675	0.54

A standard nutrient solution was then prepared and concentrated 4 times. The design of the ozonated treatments as well as the levels in mg L^{-1} of O_3 or mV of the oxidation-reduction potential are shown in Table 4.

Table 4. Description of the mixing percentage in the different experimental treatments. Irrigation water (NS_0) and fertigation with the standard nutrient solution (NS_1); $O_{0,\ 0.18,\ 0.36,\ 0.54}$: ozone at different concentrations (0, 0.18, 0.36, and 0.54 mg L^{-1}, respectively).

		Mixing Percentage (%)		
	Treatments	Nutrient Solution	Ozonated Water 0.8 mg L^{-1} ($\pm$900 mV)	Irrigation Water
Experiment 1	$NS_0\ O_0$	0	0	100
	$NS_0\ O_{0.54}$	0	75	25
	$NS_1\ O_0$	25	0	75
	$NS_1\ O_{0.54}$	25	75	0
Experiment 2	$NS_1\ O_0$	25	0	75
	$NS_1\ O_{0.18}$	25	25	50
	$NS_1\ O_{0.36}$	25	50	25
	$NS_1\ O_{0.54}$	25	75	0

The irrigation was performed manually using graduated cylinders. Although the volume of irrigation was 200 mL per plant day^{-1}, the frequency of irrigation was adjusted weekly in order to achieve a drainage percentage of around 30%.

2.4. Biometric Parameters

At the end of the experimental period, four plants per block and treatment were randomly selected and the substrate was gently removed with distilled water, avoiding the loss of roots. Each plant was divided into roots, stem, leaves, and fruits and then those organs were washed and dried with blotting paper to be weighed (fresh weight, FW) and then oven dried at 60 °C for 48 h for the determination of the dry weight (DW). These dry weights were used to determine several plant parameters, such as relative root weight ratio (RWR; root DW per unit plant DW), stem weight ratio (SWR; stem DW per unit plant DW), leaf weight ratio (LWR; leaf DW per unit plant DW), and fruit weight ratio (FWR; fruit DW per unit plant DW) as described by Garcia-Caparros et al. [21]. The total plant dry weight (TDW) was calculated as the DW sum of the roots, stems, leaves, and fruits. The fresh and dry weight of the total plant were used to calculate the relative plant water content (RWC) as indicated by Ben Amor et al. [22]. The total leaf area was determined by digitalized images of each plant using the Idrisi Selva computer program (Clark Laboratories, Worcester, MA, USA), as reported by Garcia-Caparros et al. [23].

2.5. Physiological Parameters

At the end of the experimental period, four plants were randomly selected per block and treatment for the determination of pigment concentrations. Fresh samples of leaves (0.2 g) were submerged in methanol for 24 h under dark conditions at room temperature (RT). The supernatant was removed, and the pigment concentrations were recorded spectrophotometrically using the following equations reported by Wellburn [24]. Pigment concentrations were expressed in mg g^{-1} FW.

$$\text{Chlorophyll a} = ([(15.65 \times A\_666) - (7.34 \times A\_653)] \times 15)/2 \tag{1}$$

$$\text{Chlorophyll b} = ([(27.05 \times A\_653) - (11.21 \times A\_666)] \times 15)/2 \tag{2}$$

where A_653 and A_666 are the absorbance readings at 653 and 666 nm, respectively.

The concentration of total soluble sugars and proline in leaves was determined in four plants randomly selected by treatment, following the methodology proposed by Irigoyen et al. [25]. The total soluble sugars were estimated using the anthrone reagent and the concentration of free proline was analyzed colorimetrically using ninhydrin reagent. The total soluble sugars in leaves were expressed in mg glucose g^{-1} FW and leaf proline concentration in μg g^{-1} FW.

2.6. Nutritional Parameters

The determination of nutrient concentration in the different organs assessed was performed on ground oven-dried samples. The samples were mineralized with H_2SO_4 (96%) and hydrogen peroxide (P-free) at 300 °C. Nitrogen and P concentrations in the different organs were analyzed following the protocols of Cataldo et al. [26] and Hogue et al. [27], respectively. The determination of K^+ concentration was assessed by flame spectrometry [28]. Nutrient concentrations were expressed in mg g^{-1} DW. From these determinations and with the DW measured, the plant nutrient content and partitioning in the different organs were calculated. The partitioning in the different organs was calculated by dividing the nutrient extraction of each organ by the total plant nutrient uptake.

2.7. Statistical Analysis

The experiment was analyzed as a completely randomized block design; the values obtained for each plant and each variable were considered as independent replicates. Analysis of bifactorial and unifactorial variance (ANOVA) was used and the statistical evaluations of the differences between the means of the treatments were performed by Fisher's least significant difference (LSD) tests at $p < 0.05$ using Statgraphics Plus for Windows (Statpoint Technologies Inc., Warrenton, VA, USA).

3. Results

3.1. Biometric Parameters

During the experimental period, there was neither mortality of plants nor incidence of pests or diseases noted for the treatments assessed. The fertigation with the standard nutrient solution and the application of O_3 increased significantly the total dry weight in pepper plants. For the partitioning of biomass, there were different trends among the different organs assessed. Root weight ratio increased under irrigation water and ozone application. Stem and leaf weight ratios increased under fertigation with the nutrient solution, regardless of the ozone application. The absence of nutrient solution caused an acceleration of the plant cycle, showing a higher value in fruit weight ratio (16%). Relative water content decreased under fertigation with the nutrient solution, regardless of the ozone application. Leaf area remained unchanged under the different factors assessed (Table 5).

Table 5. Effects of fertigation treatments (FT) and ozone application on *C. annuum* biometric parameters. Data are the means ± standard deviation of four samples per treatment. In a column and treatment, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

		TDW	RWR	SWR	LWR	FWR	RWC	LA
Fertigation treatments		*	*	*	*	*	*	ns
	NS₀	5.62 ± 0.34 b	0.13 ± 0.003 a	0.43 ± 0.03 b	0.27 ± 0.02 b	0.16 ± 0.01 a	7.68 ± 0.20 a	0.25 ± 0.02
	NS₁	7.17 ± 0.46 a	0.11 ± 0.002 b	0.53 ± 0.03 a	0.35 ± 0.02 a	0.00 ± 0.00 b	7.28 ± 0.19 b	0.28 ± 0.02
Ozone		*	*	ns	ns	ns	ns	ns
	O₀	5.80 ± 0.35 b	0.11 ± 0.002 b	0.47 ± 0.04	0.32 ± 0.02	0.07 ± 0.01	7.31 ± 0.20	0.25 ± 0.02
	O₀.₅₄	6.99 ± 0.47 a	0.13 ± 0.003 a	0.49 ± 0.03	0.30 ± 0.03	0.09 ± 0.01	7.56 ± 0.18	0.28 ± 0.02
Interactions		ns	ns	ns	ns	ns	ns	ns

* indicates significant differences among treatments at the $p < 0.05$ level by LSD test.

Pepper plants grown under $O_{0.18}$ and $O_{0.36}$ treatments showed the highest total dry weight (TDW). For biomass partitioning, there were significant differences between treatments. The addition of O_3 at 0.54 mg L^{-1} significantly decreased the leaf weight ratio (LWR) (Table 6). With O_3 application, there was an increase in stem weight ratio (SWR) and a decrease in root weight ratio (RWR). Relative water showed the highest value in pepper plants grown under the highest O_3 concentration. Leaf area increased under $O_{0.18}$ and $O_{0.36}$ treatments but decreased at $O_{0.54}$, showing a similar value as the control treatment (Table 6).

Table 6. Effects of increasing O_3 concentration in fertigation with the standard nutrient solution on *C. annuum* biometric parameters.

	TDW	LWR	SWR	RWR	RWC	LA
O_0	6.70 ± 0.13 c	0.37 ± 0.01 a	0.51 ± 0.005 c	0.12 ± 0.005 a	7.13 ± 0.10 b	0.27 ± 0.01 c
$O_{0.18}$	7.94 ± 0.14 a	0.36 ± 0.01 ab	0.55 ± 0.010 a	0.10 ± 0.007 b	6.28 ± 0.09 c	0.31 ± 0.01 b
$O_{0.36}$	7.87 ± 0.09 ab	0.35 ± 0.01 ab	0.53 ± 0.005 b	0.10 ± 0.006 b	7.11 ± 0.10 b	0.33 ± 0.01 a
$O_{0.54}$	7.64 ± 0.13 b	0.30 ± 0.02 b	0.53 ± 0.005 b	0.10 ± 0.007 b	7.36 ± 0.12 a	0.28 ± 0.01 c

Data are the means ± standard deviation of four samples per treatment. In a column, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

3.2. Physiological Parameters

Fertigation with the standard nutrient solution and without the application of O_3 increased the pigment concentrations and leaf proline concentrations in pepper plants. Leaf total soluble sugars remained unchanged regardless of the fertigation treatment or ozone application (Table 7). There were statistically significant differences between fertigation treatments and ozone application in pigment concentrations. The application of O_3 reduced values in Chl a and b only in the standard nutrient solution (Figure 1).

Table 7. Effects of fertigation treatments (FT) and ozone application on *C. annuum* physiological parameters. Data are the means ± standard deviation of four samples per treatment. In a column and treatment, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

		Chl a	Chl b	Chl a+b	TSS	Proline
Fertigation treatments		*	*	*	ns	*
	NS_0	2.58 ± 0.34 b	1.57 ± 0.21 b	4.15 ± 0.31 b	43.45 ± 2.71	52.20 ± 3.34 b
	NS_1	8.15 ± 0.56 a	4.36 ± 0.38 a	12.51 ± 0.81 a	45.26 ± 2.67	135.73 ± 8.75 a
Ozone		*	*	*	ns	*
	O_0	5.97 ± 0.45 a	3.37 ± 0.24 a	9.34 ± 0.73 a	44.64 ± 2.27	102.21 ± 6.57 a
	$O_{0.54}$	4.76 ± 0.37 b	2.57 ± 0.18 b	7.33 ± 0.66 b	47.06 ± 2.90	85.73 ± 5.71 b
Interactions		*	*	*	ns	ns

* indicates significant differences among treatments at the $p < 0.05$ level by LSD test.
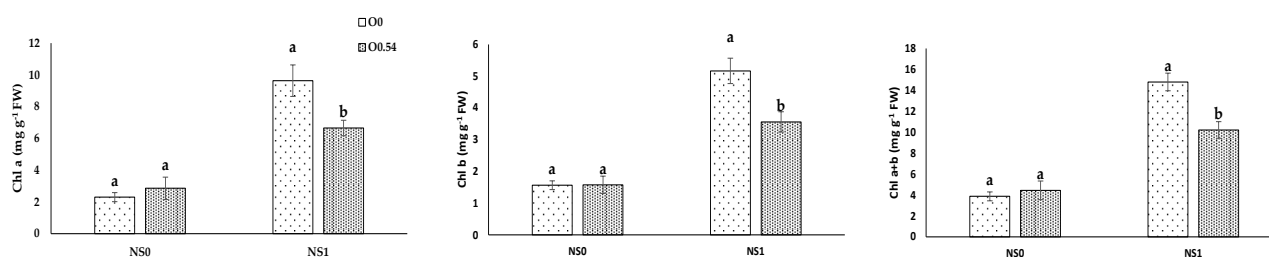
Figure 1. Interactions between fertigation treatments and ozone application on pigment concentrations. The same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

The highest value of total chlorophyll concentration was achieved in pepper plants grown under the $O_{0.18}$ treatment. There was a pigment concentration decline at higher concentrations of O_3. The lowest value of total soluble sugar concentration was for $O_{0.18}$, whereas for proline concentration this treatment showed the highest value (Table 8).

Table 8. Effect of increasing O_3 concentration in fertigation with the standard nutrient solution on *C. annuum* physiological parameters

	Chl a	Chl b	Chl a+ b	TSS	Proline
O_0	9.64 ± 0.73 a	5.17 ± 0.27 b	14.81 ± 0.49 b	44.64 ± 2.56 a	147.96 ± 8.24 ab
$O_{0.18}$	8.95 ± 0.79 a	6.49 ± 0.37 a	15.44 ± 0.57 a	33.60 ± 2.14 b	154.83 ± 7.39 a
$O_{0.36}$	7.52 ± 0.62 b	5.19 ± 0.29 b	12.71 ± 0.50 b	46.97 ± 2.89 a	121.03 ± 6.34 b
$O_{0.54}$	6.66 ± 0.35 b	3.56 ± 0.19 c	10.22 ± 0.39 c	47.88 ± 2.94 a	123.51 ± 6.22 b

Data are the means ± standard deviation of four samples per treatment at the end of the trial. In a column, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

### 3.3. Nutritional Parameters

3.3.1. Nitrogen

The total nitrogen extracted from plants (TNE) increased under fertigation with the standard nutrient solution and without the application of O_3. For the partitioning in the different organs assessed, the root nitrogen ratio (RNR) decreased under fertigation with the standard nutrient solution and the application of O_3. The stem nitrogen ratio (SNR) only decreased in pepper plants irrigated with water regardless of the O_3 application. The leaf nitrogen ratio (LNR) remained unchanged under the two factors assessed. The fruit nitrogen ratio (FNR) only increased in pepper plants irrigated with water regardless of the O_3 application, because under fertigation with the standard nutrient solution, fruits were not developed (Table 9). There were statistically significant differences between the interactions of fertigation treatments and O_3 application in the total nitrogen extracted (TNE) from plants. Under water irrigation there was no significant difference between ozone applications (Figure 2).

Table 9. Effects of fertigation treatments (FT) and ozone application on total nitrogen extracted from plants (TNE) (mg plant^{-1}) and their distribution in root (RNR), stem (SNR), leaf (LNR), and fruit (FNR) in *C. annum* at the end of the experimental period. Data are the means ± standard deviation of four samples per treatment. In a column and treatment, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

		TNE	RNR	SNR	LNR	FNR
FT		*	*	*	ns	*
	NS_0	49.70 ± 2.85 b	0.15 ± 0.01 a	0.29 ± 0.02 b	0.39 ± 0.03	0.16 ± 0.01 a
	NS_1	360.88 ± 24.75 a	0.12 ± 0.01 b	0.55 ± 0.04 a	0.34 ± 0.03	0.00 ± 0.00 b
Ozone		*	*	ns	ns	ns
	O_0	227.03 ± 16.14 a	0.15 ± 0.01 a	0.41 ± 0.04	0.33 ± 0.03	0.08 ± 0.01
	$O_{0.54}$	193.55 ± 13.77 b	0.12 ± 0.01 b	0.43 ± 0.04	0.35 ± 0.03	0.08 ± 0.01
Interactions		*	ns	ns	ns	ns

* indicates significant differences among treatments at the $p < 0.05$ level by LSD test.
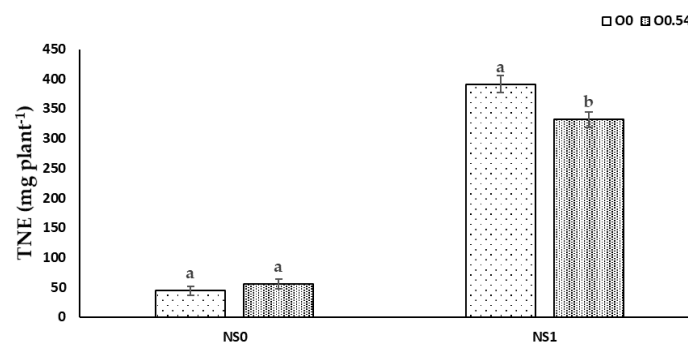
Figure 2. Interactions between fertigation treatments and ozone application on total nitrogen extracted (TNE) from plants. The same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

The total N extracted by plants was significantly higher under $O_{0.18}$ treatment and decreased with the increase in O_3 concentration. The lowest value of RNR in pepper plants was presented at $O_{0.18}$ treatment. Pepper plants grown under $O_{0.36}$ showed the lowest value of SNR and the highest value of LNR (Table 10).

Table 10. Effects of increasing O_3 concentration in fertigation with the standard nutrient solution in total nitrogen extracted from plants (TNE) (mg $plant^{-1}$) and their distribution in root (RNR), stem (SNR), and leaf (LNR) in *C. annum* at the end of the experimental period.

	TNE	RNR	SNR	LNR
O_0	390.09 ± 23.68 b	0.13 ± 0.020 a	0.54 ± 0.04 a	0.33 ± 0.02 c
$O_{0.18}$	438.45 ± 21.24 a	0.07 ± 0.005 b	0.55 ± 0.04 a	0.41 ± 0.03 ab
$O_{0.36}$	337.70 ± 19.65 c	0.11 ± 0.010 a	0.46 ± 0.03 b	0.43 ± 0.03 a
$O_{0.54}$	331.67 ± 20.01 c	0.11 ± 0.010 a	0.55 ± 0.04 a	0.34 ± 0.02 bc

Data are the means $\pm$ standard deviation of four samples per treatment. In a column, the same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

3.3.2. Phosphorus

Total phosphorus extracted from plants (TPE) increased under fertigation with the standard nutrient solution and without the application of O_3. Regarding partitioning in the different organs assessed, the root phosphorus ratio (RPR) decreased under fertigation with the standard nutrient solution and without the application of O_3. The stem phosphorus ratio (SPR) showed an opposite trend compared to RPR. The leaf phosphorus ratio (LPR) increased under fertigation with the standard nutrient solution and the application of O_3. The fruit phosphorus ratio (FPR) only increased in pepper plants irrigated with water regardless of the O_3 application, because under the fertigation with the standard nutrient solution, fruits were not developed (Table 11). There were significant interactions between fertigation treatments and ozone applications in the total phosphorus extracted (TPE) from plants, SPR, and LPR (Figure 3). In the TPE, under O_3 supply the increase in total P extraction was significantly higher than without O_3 supply when water was applied, but in the case of the standard nutrient solution, the trend was the opposite. Irrigation with water did not result in differences in SPR related to O_3 supply, but with the standard nutrient the highest O_3 concentration reduced SPR. In LPR, the value was higher under irrigation water and without O_3 supply, whereas in the case of the standard nutrient solution, the trend was the opposite.

Table 11. Effects of fertigation treatments (FT) and ozone application on total phosphorus extracted from plants (TPE) (mg $plant^{-1}$) and their distribution in root (RPR), stem (SPR), leaf (LPR), and fruit (FPR) in *C. annum* at the end of the experimental period. Data are the means $\pm$ standard deviation of four samples per treatment. In a column, the same letter (a–b) indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

		TPE	RPR	SPR	LPR	FPR
FT		*	*	*	*	*
	NS_0	29.25 ± 1.57 b	0.16 ± 0.01 a	0.27 ± 0.02 b	0.32 ± 0.02 b	0.23 ± 0.02 a
	NS_1	49.73 ± 2.16 a	0.12 ± 0.01 b	0.52 ± 0.04 a	0.37 ± 0.02 a	0.00 ± 0.00 b
Ozone		*	*	*	*	ns
	O_0	42.10 ± 2.09 a	0.11 ± 0.01 b	0.43 ± 0.03 a	0.32 ± 0.02 b	0.12 ± 0.01
	$O_{0.54}$	26.88 ± 1.47 b	0.17 ± 0.01 a	0.36 ± 0.03 b	0.38 ± 0.03 a	0.12 ± 0.01
Interactions		*	ns	*	*	ns

* indicates significant differences among treatments at the $p < 0.05$ level by LSD test.

The total P extraction was significantly higher in pepper plants grown under $O_{0.18}$ treatment, and decreased with increasing O_3 concentrations. Pepper plants grown under $O_{0.54}$ treatment showed the highest value of RPR, whereas the control treatment showed the

lowest value. Under O_0 and $O_{0.18}$ treatments, pepper plants showed higher values of SPR than $O_{0.36}$ and $O_{0.54}$ treatments. Nevertheless, LPR showed an opposite trend (Table 12).
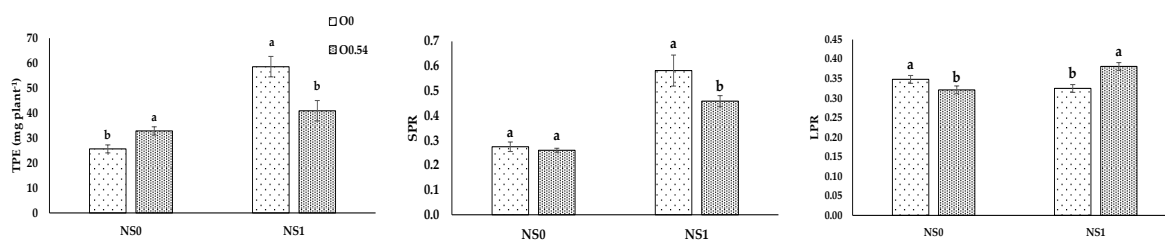

Figure 3. Interactions between fertigation treatments and ozone application on total phosphorus extracted (TPE) from plants, SPR, and LPR. The same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

Table 12. Effects of increasing O_3 concentration in the fertigation with the standard nutrient solution in total phosphorus extracted from plants (TPE) (mg plant^{-1}) and their distribution in root (RPR), stem (SPR), and leaf (LPR) in *C. annum* at the end of the experimental period.

	TPE	RPR	SPR	LPR
O_0	58.55 ± 2.34 b	0.09 ± 0.01 c	0.58 ± 0.04 a	0.33 ± 0.01 b
$O_{0.18}$	63.32 ± 1.74 a	0.13 ± 0.01 b	0.55 ± 0.03 a	0.34 ± 0.01 b
$O_{0.36}$	53.23 ± 2.06 c	0.13 ± 0.01 b	0.48 ± 0.02 b	0.38 ± 0.02 a
$O_{0.54}$	40.91 ± 1.89 d	0.16 ± 0.01 a	0.46 ± 0.02 b	0.38 ± 0.02 a

Data are the means $\pm$ standard deviation of four samples per treatment. In a column, the same letter (a–c) indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

3.3.3. Potassium

Total potassium extracted from plants (TKE) increased under fertigation with the standard nutrient solution and with the application of O_3. For partitioning in the different organs assessed, the root potassium ratio (RKR) decreased under fertigation with the standard nutrient solution regardless of the O_3 application. The stem potassium ratio (SKR) increased in pepper plants fertigated with the standard nutrient solution and with the application of O_3. The leaf potassium ratio (LKR) decreased under fertigation with the standard nutrient solution and the application of O_3. The fruit potassium ratio (FKR) only increased in pepper plants irrigated with water regardless of the O_3 application because under the fertigation with the standard nutrient solution, fruits were not developed (Table 13). There were significant interactions between fertigation treatments and ozone application in total potassium extracted (TKE) from plants, RKR, SKR, and LKR (Figure 4). Under water application similar TKE values were found, but the values were higher with O_3 in the standard nutrient solution. The root potassium ratio (RKR) and SKR were significantly higher when nutrient solution was applied and under O_3 supply. The leaf potassium ratio (LKR) showed an opposite trend considering fertigation with the nutrient solution without O_3 supply.

Total K extraction was significantly higher in pepper plants grown under $O_{0.18}$ and $O_{0.36}$ treatments, and decreased with increasing O_3 concentrations; the control treatment showed the lowest value. The lowest value for RKR and SKR was found in pepper plants grown under the control treatment (0 mg L^{-1} of O_3). The LKR was highest for O_0 and $O_{0.36}$ treatments (Table 14).

Table 13. Effects of fertigation treatments (FT) and ozone application on total potassium extracted from plants (TKE) (mg plant^{-1}) and their distribution in root (RKR), stem (SKR), leaf (LKR), and fruit (FKR) in *C. annum* at the end of the experimental period. Data are the means ± standard deviation of four samples per treatment. In a column, the same letter (a–b) indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

		TKE	RKR	SKR	LKR	FKR
FT		*	*	*	*	*
	NS$_0$	208.16 ± 15.26 b	0.09 ± 0.01 a	0.34 ± 0.03 b	0.44 ± 0.02 a	0.12 ± 0.01 a
	NS$_1$	389.49 ± 20.35 a	0.06 ± 0.01 b	0.52 ± 0.04 a	0.40 ± 0.01 b	0.00 ± 0.00 b
Ozone		*	ns	*	*	ns
	O$_0$	279.34 ± 19.38 b	0.07 ± 0.02	0.38 ± 0.03 b	0.48 ± 0.03 a	0.12 ± 0.01
	O$_{0.54}$	318.31 ± 15.17 a	0.09 ± 0.02	0.47 ± 0.04 a	0.37 ± 0.03 b	0.13 ± 0.01
Interactions		*	*	*	*	ns

* indicates significant differences among treatments at the $p < 0.05$ level by LSD test.
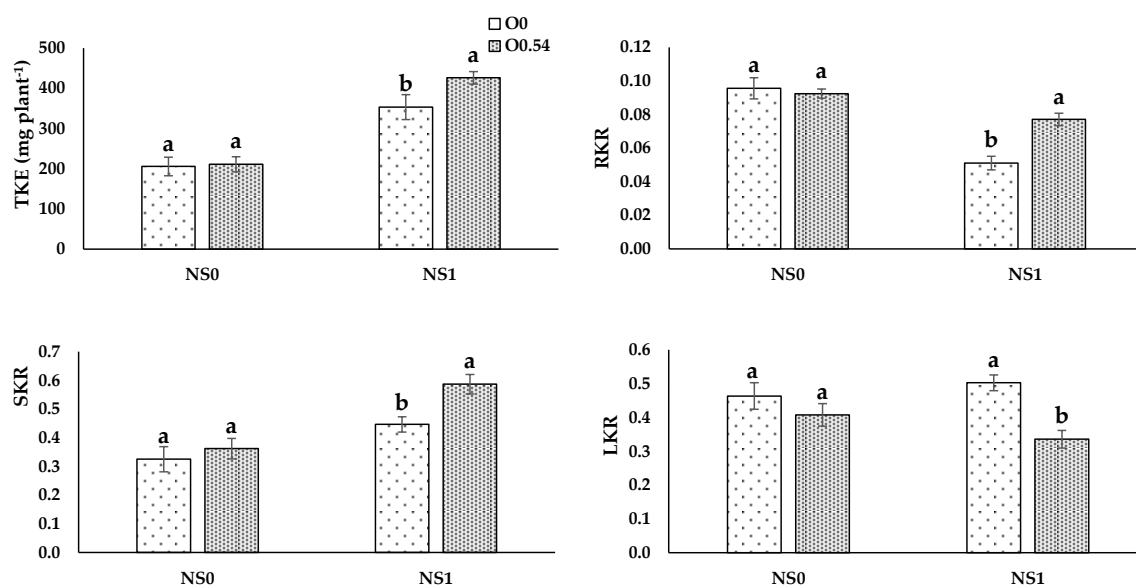

Figure 4. Interactions between fertigation treatments and ozone application on total potassium extracted (TKE) from plants, RKR, SKR, and LKR. The same letter indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

Table 14. Effects of increasing O$_3$ concentration in the fertigation with the standard nutrient solution in total potassium extracted from plant (TKE) (mg plant^{-1}) and their distribution in root (RKR), stem (SKR), and leaf (LKR) in *C. annum* at the end of the experimental period.

	TKE	RKR	SKR	LKR
O$_0$	353.17 ± 17.19 c	0.05 ± 0.007 b	0.45 ± 0.04 b	0.50 ± 0.04 a
O$_{0.18}$	484.78 ± 20.45 a	0.06 ± 0.005 ab	0.61 ± 0.04 a	0.34 ± 0.03 b
O$_{0.36}$	455.79 ± 23.49 ab	0.07 ± 0.006 ab	0.60 ± 0.04 a	0.53 ± 0.04 a
O$_{0.54}$	425.82 ± 24.28 b	0.08 ± 0.005 a	0.59 ± 0.05 a	0.34 ± 0.03 b

Data are the means ± standard deviation of four samples per treatment. In a column, the same letter (a–c) indicates no significant differences among treatments at the $p < 0.05$ level by LSD test.

4. Discussion

Coll et al. [29] defined as "unfavorable conditions" those in which the environmental conditions deviate from those considered optimal, and plants modify their development to adapt to them, producing enzymes or accelerating their development. These unfavorable conditions are known as biotic or abiotic stress.

Under stress conditions such as high temperature [30], waterlogging [31], salinity [32], and hydric and nutrient deficiencies [25], plants must change their physiology and increase their efficiency so as to use few nutrients available to fulfill their development cycles as occurs under O_0.

The supply of O_3 dissolved in irrigation water led to an autolysis process in the matrix substrate, generating active oxygen species (H_2O_2, OH^-, O_2^-) [33] and an increase in pH [34]. The chemical autolysis is potentiated because O_3 reacts with molecules containing nitrogen, hydrogen, chlorine, or bromine. Moreover, there should not be transference to the environment because O_3 solubility in water is in the range of 780 mg L^{-1} at 10 °C to 270 mg L^{-1} at 40 °C, higher that our tested concentrations. For these reasons, we suggest that under the conditions assessed in our experiment, pepper plants should have higher O_2 availability. Sloan and Engelke [35] reported similar conditions in *Agrostis stolonifera* using aerated nutrient solution and O_3.

Considering O_2 concentration in the nutritive solution, studies carried out applying low concentrations of O_2 in the nutrient solution in corn plants showed a decrease in biomass [36], especially in root biomass [37]. These results could be considered similar to O_0 compared to $O_{0.54}$ in the first experiment. Although in O_0 there were no anoxic conditions that may cause a shortage of O_2, its concentration was lower than in $O_{0.54}$.

Suyantohadi et al. [38] reported beneficial effects applying high levels of dissolved O_2 (from 20 to 30 mg L^{-1}) in hydroponically grown lettuce. Experiments with cucumbers and peppers had relatively little success in improving the yield of fruits exposed to an increase in dissolved O_2 in the nutrient solution [39]. In addition, O_2 concentrations higher than 30 mg L^{-1} generated detrimental production in tomato under a hydroponic system [40].

Considering O_3 supplies, Kobayashi et al. [41], using O_3 microbubbles at 0.2 ppm in the nutrient solution, reported no adverse effects on lettuce growth. Similar results were reported by Zheng et al. [42] (2–4 mg L^{-1}) in lettuce and by Ohashi-Kaneko et al. [43] (0–10 mg L^{-1}) in tomato plants. Nevertheless, Najarian et al. [44] reported a higher growth of cucumber plants with an O_3 dosage of 0.5 mg L^{-1}. Similarly, irrigation with ozonated water at 0.5 mg L^{-1} increased the fresh weight of bok choy and Chinese spinach [45]. This increase in biomass can be related to the enhancement of root respiration by the presence of ozonated water [46].

The data obtained in our experiment show an increase in biomass especially accumulated in the stem fraction of pepper plants through the application of O_3 in the nutritive solution treatments, in agreement with the results reported by Graham et al. [18] in tomato. Nevertheless, the highest total dry weight in the second experiment was associated with the $O_{0.18}$ and $O_{0.36}$ treatments but not at the highest concentration, which can be associated with the higher sensitivity to elevated O_3 reported by Wittig et al. [47].

In our experiment, pepper plants irrigated only with water showed the highest relative water content. These results agree with those proposed by Morales et al. [48], who compared water and nutrient solution treatments and reported an osmotic effect caused by an increase in salts due to the presence of fertilizers, which can restrict the root water uptake and affect the plant water status. On the same hand, Parra-Terraza et al. [49] reported that if there is an increase in nutrient concentration in the nutrient solution, the osmotic potential increases in absolute value, decreasing the free energy, and consequently the uptake processes of water and some nutrients are affected.

With respect to O_3 supply, there were no significant differences in relative water content in pepper plants, but when O_3 concentration increased in the nutrient solution, there was also an increase in RWC in pepper plants. After reviewing previous literature, we found no references about the modifications of the water status of one species after the application of ozonated water. Wilkinson and Davies [50] reported that under a combined O_3 stress caused by atmospheric concentrations of 60–80 ppb and drought conditions, there was an ethylene-dependent reduction in the sensitivity of stomata to abscisic acid (ABA) and repressed stomatal closure, which would mean a loss of water and therefore lower values of RWC. Nevertheless, under well-watered conditions, O_3 often reduces stomatal

aperture [47,51]. Therefore, considering that our experiment was conducted under well-watered conditions, the results reported in $O_{0.54}$ treatment may be due to higher sensitivity to elevated O_3.

Pepper plants did not show significant differences in leaf area under water irrigation or fertigation with a standard nutrient solution with or without the application of O_3. Nevertheless, the increase in O_3 supplies increased leaf area but at $O_{0.54}$, leaf area in pepper plants showed a decline. These results were in line with Chan et al. [45], who reported that the irrigation by ozonated water for one month increased the leaf area of bok choy and Chinese spinach (doses from 0.5 mg L^{-1} to 1.5 mg L^{-1}). In addition, Peykanpour et al. [46] reported that the increase in leaf area in cucumber plants was associated with the increase in O_3 concentration from 0 to 1 mg L^{-1}. Nevertheless, Najarian et al. [44] reported an increase in leaf area in cucumber plants up to 0.5 mg L^{-1} O_3 concentration.

As far as physiological parameters were concerned, in our experiment, the application of O_3 resulted in an increase in Chl b and Chl a+b under $O_{0.18}$, in line with the results proposed by Sloan and Engelke [35], who reported a significant increase in chlorophyll concentration in *Agrostis stolonifera* under the application of O_3. Moreover, our results for pigment concentrations in pepper plants under $O_{0.36}$ were in line with the results proposed by Chan et al. [45], who noted not significant differences in chlorophyll concentration under the application of ozonated water (doses from 0.5 mg L^{-1} to 1.5 mg L^{-1}) in vegetable species (choy sum, bok choy, green mustard, and Chinese spinach) in the vegetative stage. Similarly, Martínez-Sánchez and Aguayo [19] reported that the application of ozonated water affected neither the photosynthesis rate nor the development of grafted watermelon seedlings. In addition, Paniagua [52] reported no effects in Chl a and Chl a+b associated with the action of this oxidizing compound in *Cordyline fruticosa* plants var. "Red Edge" fertigated with different mixtures of H_2O_2. In our experiment, we reported a reduction in Chl a, Chl b, and Chl a+b concentration under $O_{0.54}$, which can be related to the possible O_3 transference to the environment associated with a significant decrease in chlorophyll concentration, as has been reported for wheat [53] and lettuce [54] under environmental pollution.

The concentration of total soluble sugars in leaves can be considered the balance of bioassimilated generation processes and respiration losses. Sugars in turn are important substrates in the respiration process, which ensure plants have sufficient energy necessary to increase the intensity of some metabolic processes activated under stress factors [55]. In previous literature, Nicoletto et al. [56] reported no effect of O_3 supply on leaf total soluble sugars in a forcing closed system process of *Cichorium intybus*. Similarly, the application of O_3 in gaseous form to the soil did not result in changes in leaf total soluble sugars in *Fagus sylvatica*. These results agree with the data obtained from our experiment, except for the case of $O_{0.18}$, which presented a significantly lower leaf total soluble sugars concentration. This fact can be associated with a differential carbon partitioning and also due to the dilution effect since $O_{0.18}$ showed the highest TDW [57].

Proline is a biochemical indicator related to stress tolerance in biotic and abiotic stress [58]. For instance, the intervention of this osmolyte has been reported in plants grown under a water deficit [59,60], saline conditions [4], changing light conditions [61], heavy metal concentrations [62], and with pests and diseases [63].

Although it has been reported that under nutrient imbalances, including mineral toxicities and deficiencies of nitrogen, phosphorus, or potassium, there is an increase in proline concentration [64], our experiment showed a decline in proline concentration in pepper plants irrigated only with water.

A significant decrease in leaf proline concentration was evident at higher O_3 concentrations. In the literature, there are no references about the effects of the supply of O_3 in fertigation on leaf proline concentration. Nevertheless, under O_3 tropospheric pollution, El-Khatib [65] reported an increase in leaf proline concentration in several species such as *Senecio vulgaris*, *Malva parviflora*, *Sonchus oleraceus*, *Medicago sativa*, and *Melilotus indicus* under increasing O_3 concentrations (from 50 ppb to 100 ppb). The decrease in leaf proline concentration in this study may suggest that the application of O_3 in the standard nutrient

solution did not result in oxidative stress in pepper plants, which is associated with the increase in different osmolytes such as proline.

It is well known that N, P, and K are essential for the adequate development of a crop, the latter especially in the stages of flowering and fruit setting. Therefore, these macronutrients are consumed in high amounts and if there is any deficiency of one of them, there is a reduction in crop yield [66], as happened in our treatment with only water irrigation. The lower N, P, and K extraction in plants grown under water irrigation against fertigation with the standard nutrient solution was in line with the findings of Marschner [67], mainly based on the availability or not for nutrient uptake. In addition, sterilization with ozonated water could increase the water and mineral uptake by the root system of the plant [43]. Nevertheless, Martínez-Sánchez and Aguayo [19] used ozonated fertigation (750 mV) in *Capsicum* seedlings and reported no differences in leaf N concentration, but significantly lower concentrations of P and K compared to the control treatment without O_3. The results of our experiment showed lower plant N uptake but a similar leaf nitrogen ratio under increasing O_3 concentrations. Plant total P extraction showed a significant decrease compared to the control treatment, in line with the results reported by Martínez-Sánchez and Aguayo [19], who noted the same trend. Nevertheless, the increase in plant total K extraction showed an opposite trend compared with the results obtained by Martínez-Sánchez and Aguayo [19].

For O_3 concentration, the highest N, P, and K extractions were evident in pepper plants grown under $O_{0.18}$ treatment. Potassium extraction increased from O_0 to $O_{0.36}$, but at the highest concentration (0.54 mg L^{-1}) showed a decrease compared to $O_{0.18}$, which can be associated with the fact that K is the most sensitive nutrient to the lack of O_2 due to its efflux to the medium [68].

Nutrient uptake processes are linked to H^+-pumps via cotransport, antiport, and uniport mechanisms, which are related to the O_2 availability in the nutrient solution, as reported by Clarkson and Lüttge [69]. Therefore, this fact can be the reason for the increase in TNE, TPE, and TKE under $O_{0.18}$ treatment compared to O_0 treatment. Nevertheless, at higher O_3 concentrations, an antagonistic effect may occur as a consequence of a reduction in transpiration and an impairment of the active transport of N, P, and K, which can be responsible for disruptions in the nutrient transport from roots to shoots [70], as occurs when comparing $O_{0.36}$ to $O_{0.54}$, since we noted an increase in RNR, RPR, and RKR and a decrease in stem and leaf ratios.

5. Conclusions

The O_3 supply via fertigation increased growth evaluated as biomass produced but showed lower root development. This higher biomass may be associated with higher plant N and K uptake. The recommended dosage of O_3 is from 0.18 to 0.36 mg L^{-1}. The increase in stem and leaf weight ratios can be ascribed to a higher leaf area and an increase in chlorophyll concentration. Moreover, a higher synthesis of bioassimilates favoring plant growth may be related to the higher values of N, P, and K uptake. The results obtained with the O_3 supply in fertigation in pepper plants in these experiments are very encouraging. Nevertheless, higher dosages such as 0.54 mg L^{-1} of O_3 should be studied in more depth in order to assess oxidative and/or phytotoxic damage associated with the supply of O_3. Comparative studies with other oxidizing compounds and dissolved O_3 supply should be conducted in order to check their implementation in intensive greenhouse horticulture.

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