horticulturae

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Mitigation of Chilling Stress by Ozone Pretreatment and Acclimation of Sweet Pepper Grown under Unheated Greenhouse Conditions

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Citation: Sharaf-Eldin, M.A.; Etman, A.A.; Yassin, A.M.; Elsayed, S.; Scholz, M.; Yaseen, Z.M. Mitigation of Chilling Stress by Ozone Pretreatment and Acclimation of Sweet Pepper Grown under Unheated Greenhouse Conditions. *Horticulturae* **2022**, *8*, 1189. https://doi.org/10.3390/horticulturae8121189

Academic Editor: Luigi De Bellis

Received: 17 November 2022
Accepted: 9 December 2022
Published: 13 December 2022

Abstract: Ozone is an important air pollutant that causes many challenges for human health, such as lung diseases. The negative impacts of exogenous ozone on cultivated plants have been discussed in several publications, but the positive impacts are less investigated. The current study is an attempt to answer the following question: is there any positive contribution of ozone pretreatment in growing plants under stress? Plants grown in unheated plastic greenhouses suffer from cold stress during the winter when the temperature drops to 5–10 °C. This stress can also be enhanced under saline soil conditions in arid regions. Treatments involving different ozone application methods (seed priming and seedling foliar application) and cold pretreatment (4 °C for 36 h) were compared with untreated controls of two sweet pepper hybrids (Zidenka and Lirica) in an unheated plastic greenhouse. In general, the Lirica hybrid outperformed the Zidenka hybrid in growth and fruit yield and showed good adaptation to cold stress resulting from ozone treatment through foliar application on seedlings. Moreover, cold treatment was associated with higher values of the studied parameters compared to the control (untreated). A concentration of 20 ppm of ozone in the first season and as 30 ppm of ozone in the second season increased plant leaf area, proline content, and APX activity, all of which improved the plant's defense mechanisms against cold stress. These benefits contributed to high fruit sets; early, total, and marketable fruit yields; and fruit quality under cold stress. The highest yield (16.52 kg m^{-2}) was attained with 30 ppm ozone applied as a seedling spray, compared with 10.07 kg m^{-2} in the control. Therefore, the foliar application of ozone up to 30 ppm can be recommended for sweet pepper hybrids of Lirica under unheated plastic greenhouse conditions. Further investigations are needed to study the interaction of combined ozone and cold stress, as well as various levels of soil salinity.

Keywords: proline; ascorbate peroxidase; membrane permeability; yield and quality; cold pretreatment

1. Introduction

Global climate change has a negative impact on the agricultural sector, especially in arid regions [1–3]. Cold temperatures are a serious factor limiting the survival, productivity, and geographical distribution of plants in large areas worldwide [4,5]. Exposure to a low, non-lethal temperature usually has a significant adverse effect on the biochemical and physiological characteristics of plants through photosynthesis, antioxidant activity, uptake of water and nutrients, plant growth and productivity, and the quality of many economically important crops, such as fruits of loquat [6], zucchini [7], tomato [1], and pepper [2]. The inability to increase membrane fluidity and suppress photosynthetic processes results in membrane leakiness and negatively affects pepper productivity [3,8]. As reported by El-Aidy and Sharaf-Eldin [3], in the same experimental location and under the same conditions as those of the current study, low temperature in an unheated plastic greenhouse during the winter limited growth and significantly reduced fruit yield and quality, causing significant economic loss of sweet pepper in Egyptian.

Environmentally friendly approaches for overcoming biotic and abiotic stresses to crops play a crucial role in improving plant production and quality. One of the most common and effective approaches is plant breeding [9]. Additionally, protected cultivation successfully protects plants from various kinds of stresses in several regions [10]. Changing agricultural practices is essential for managing stress. Grafting is considered a valuable practice for increasing crop yield under stress conditions [11]. In a recent study, Consentino et al. [11] reported that the combined application of grafting and biostimulants (growth-promoting bacteria from *Azospirillum brasilense* DSM 2298) is a sustainable agronomic practice that significantly enhanced eggplant growth, yield, and quality under protected cultivation conditions.

The production of vegetable crops under stress is a challenge for sustainable agricultural systems under greenhouse conditions [12]. In places with mild, sunny winters, such as the Mediterranean, it is common to grow crops that require elevated temperatures in unheated greenhouses in the winter; however, the plants still suffer from cold stress, which negatively affects production. This strategy can reduce high cost associated with heating energy, especially given the high energy expenses brought on by the war between Russia and Ukraine. However, this strategy would be more effective if combined with adaptation techniques for cold stress. Cold adaptation requires early exposure to non-lethal low temperatures for a brief period [13]. Modifications in morphological, physiological, and biochemical processes involving antifreeze proteins, cell membrane inflexibility, compatible solutes, and epigenetic mechanisms enhance cold stress tolerance [14]. Moreover, antifreeze activity (enzymatic antioxidants, phenolics, and proline) and molecular changes in plants can result in an acclimation response that is characterized by a increased ability to resist low-temperature stress [15].

Ozone (O_3), a natural gas in the atmosphere, is considered one of the most damaging air pollutants [16] to cultivated plants at the ground level (30–100 $\mu g\ m^{-3}$) because of its phytotoxicity. It may be toxic in agricultural and rural regions at high concentrations (300–800 $\mu g\ m^{-3}$) [17]. Many adverse impacts of ozone on plant growth (e.g., reducing biomass production, inhibiting photosynthetic processes, and the formation of oxidative stress) have been reported for many crops, such as tomato, spinach, chard [18], common bean [19], maize [20], soybean [21], and wheat [22]. On the other hand, ozone could be used as an antimicrobial agent, owing to its strong oxidative ability in both aqueous and gaseous phases, and is useful for fruit preservation because it has no side effects on nutritional values and quality of products [23]. Hence, ozone has a distinguished role in extending the shelf life of postharvest fruits and vegetables [24] such as strawberry [25], tomato [26], and cantaloupe [16].

In addition to its crucial role in enhancing secondary metabolites and bioactive properties, the role of ozone in promoting plant growth and yield against many pathogens and abiotic stress has attracted considerable attention [27]. Some studies have reported on the role of exogenous ozone in pomegranate under stressful conditions, such as salinity [28], drought on *Phillyrea angustifolia* [29], and cold stress on cantaloupe [20]. When plants are exposed to nontoxic doses of ozone, their phytochemical parameters, including the antioxidant defense system and bioactive compounds, change as a typical initial stress response [30]. This modification of the plant biochemical system can contribute to mitigation of other kinds of stresses in the future.

Sweet pepper (*Capsicum annuum* L.) is one of the most important horticultural plants, owing to its nutritional value (ascorbic acid or vitamin C, antioxidants, and carotenoids), color, and flavor [31]. Pepper plants originate in tropical or subtropical regions of the world, so they require an elevated temperature for development that ranges from 25 to 30 °C [32]. Thus, sweet pepper commonly suffers from chilling stress in the winter season, and dramatic damage can occur in association with salt stress under in unheated plastic greenhouses in the winter season. Seed priming and foliar antistressors are crucial methods for mitigating stress on cultivated plants such as salinity stress [33] and water-deficit stress [34]. Several antistressors have been used in seed priming to overcome many stresses, such as potassium nitrate or salicylic acid under water-deficit stress [34] and salicylic acid under salinity–alkalinity stress [33]. Methyl salicylate was applied for the production of sweet pepper under stress [2]. Moreover, exogenous zeaxanthin was used on pepper seedlings to mitigate chilling injury.

The first published data about the positive role of ozone in improving sweet pepper resistance to cold stress only considered the plant growth in the seedling stage [8]. However, no articles on the role of ozone in the production of sweet pepper under cold conditions have been published to date. Nevertheless, such high stress commonly occurs in unheated plastic greenhouses in arid regions. Hence, the aim of this study is to answer the following questions: Which method is better for sweet pepper production in an unheated plastic greenhouse under cold stress: ozone or cold pretreatments with seed priming and/or seedling spraying? Which application method is better: seed priming or foliar spraying of seedling? Is the growth stage a factor in determining the appropriate application of ozone or chilling for sweet pepper acclimation? This study was carried out to evaluate the tolerance of sweet pepper hybrids to cold stress and saline soil conditions through ozone and cold pretreatments of seed priming or foliar spray of seedlings in an unheated plastic greenhouse. The impacts of different treatments on physiological and biochemical characteristics, as well as productivity, during plant growth were also investigated.

2. Materials and Methods

This work was carried out on two sweet pepper hybrids (Zidenka and Lirica) under plastic greenhouse conditions at the International Protected and Smart Agriculture Center, Faculty of Agriculture, Kafrelsheikh University, Egypt, during the two seasons of 2017/2018 and 2018/2019.

2.1. Climatic and Microclimatic Conditions

During the experimental period from September until June in both growing seasons, the maximum and minimum air temperatures for the inside and outside of the plastic greenhouse were recorded using RH/Temp data loggers (model BESANTEK BST-DL13, Nanjing, Jiangsu, China), as presented in Figure 1.

2.2. Treatments

The treatments included:

(A) Two sweet pepper hybrids (Zidenka and Lirica)

The plants were bought from the seed company Rijk Zwaan (The Netherlands). Healthy seeds with consistent in size and shape were chosen and sterilized for 5 min with 1% sodium hypochlorite.

(B) Four concentrations of ozonated water were compared to cold treatment and an untreated control.

An ozone generator (Matra—GL—2186, China) was used to make ozone-enriched water with different concentrations. Four O_3 concentrations (1, 5, 10, and 20 mg L^{-1}) were applied for the first season. However, the lower concentrations (1 and 5 mg L^{-1}) were replaced by two higher concentrations (30 and 60 mg L^{-1}) in the second season. Ozone concentrations were compared to cold stress treatment and an untreated control during both growing seasons.

(C) Two application methods in two separate experiments (seed soaking and seedling spraying)

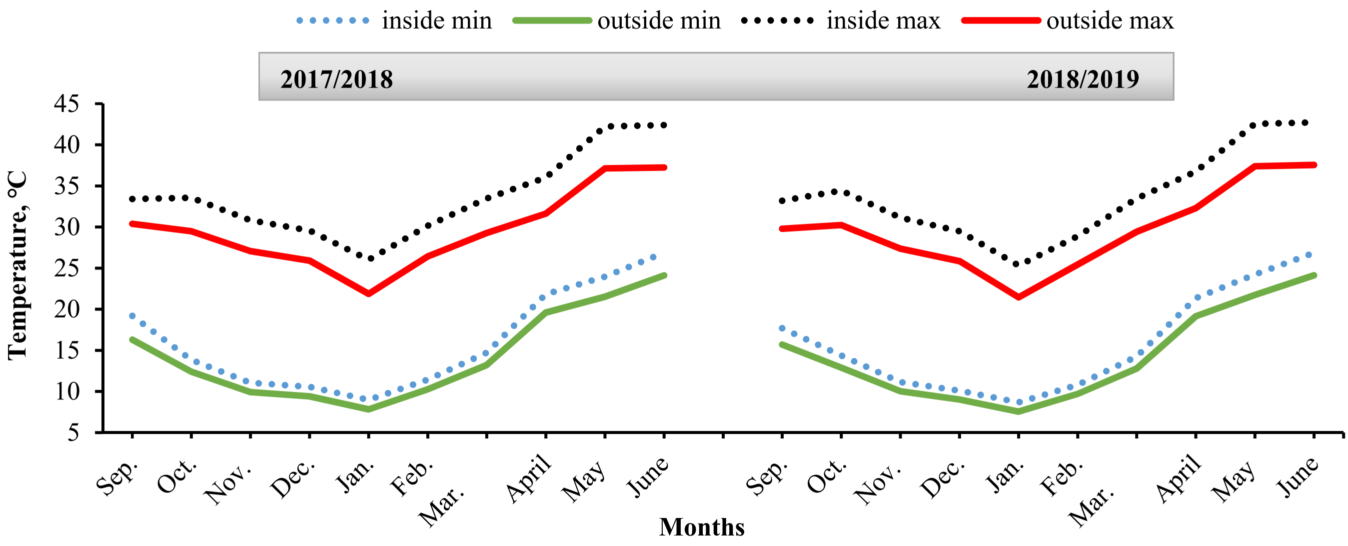


Figure 1. Maximum and minimum temperature for the inside and outside of the plastic greenhouse during the seasons of 2017/2018 and 2018/2019.

Seed soaking treatment:

The seeds were soaked in distilled water for 24 h and dried for 30 min at room temperature. Then, they were deep-soaked in the abovementioned concentrations of concentrated O_3 solution at room temperature for 1 h, followed by washing in running tap water for about 1 min (to avoid damage by ozone), were left to dry on the surface of paper towels under ambient conditions (22 °C and 65% relative humidity), then planted. For untreated seeds (control), the same procedures were followed, but water alone was used in place of the ozone solution. After germination, a chilling treatment was conducted on the seeds at 4 ± 1 °C for 36 h in the light [35]. The treated seeds were sown in seedling trays, which were filled with a mixture of peat moss and vermiculite (1:1 *v*/*v*). Untreated seeds (control) were sown at the same time as the treated seeds. In brief, this experiment comprised three groups. The first group was the untreated control (without ozone treatment or cold treatment). The second group was treated with ozone concentrations. The third group was subjected to cold treatment to induce cold resistance.

Seedling spray treatment:

Another separate experiment was conducted on another group of seeds. Untreated seeds were sown in seedling trays until they grew three real leaves. In this stage, the studied O_3 concentrations were foliar-sprayed as a seedling treatment. The seedlings were sprayed with O_3 solutions three times within three-day intervals. The chilling treatment was conducted at 4 ± 1 °C for 36 h in the light at the same age as the seedlings were treated with O_3. Untreated seedlings were considered the control. Thus, this experiment contained comprised groups of seedlings; untreated control (without cold or ozone treatments), ozone as a foliar spray, and cold treatment.

Pepper seeds were sown in seedling trays on 25 September in the first season and 5 September in the second season. After sowing, the trays were covered by black plastic for 3–4 days, then removed. Subsequently, the common practices of irrigation, fertilization, and pest management were performed in the nursery. The seedlings from each of the previously mentioned experiments were transplanted separately to same greenhouse. Each experiment had a separate untreated control (without ozone or cold treatment). The greenhouse comprises 1200 m^2 of land, with a length of 40 m, a width of 30 m, and a height of 3.5. The greenhouse was covered by a 200 µm thick polyethylene plastic film, and the he ventilation openings were covered by an insect-proof net sheet. The treatments were carried out on seeds and seedlings. The evaluated parameters were determined in mature plants under greenhouse conditions to consider the plant behavior during the production period.

2.3. Studied Data

2.3.1. Plant Leaf Area

Thirty and sixty days after transplanting, vegetative growth parameters were measured, i.e., leaf area per plant (m^2), using a portable leaf area meter (model LI-3000A, Lincoln, NE, USA).

2.3.2. Plant Biochemical and Physiological Parameters

Some biochemical assessments were conducted three times: (1) at the beginning of cold stress, (2) during the extreme cold stress period, and (3) after the cold period (i.e., after 30, 60, and 90 days, respectively).

(A) The relative chlorophyll content (SPAD) or green color content in the first fully expanded leaves was determined (without destroying them) using an SPAD501 apparatus (Minolta Corp, Ramsey, NJ, USA) for greenness measurements.

(B) Membrane permeability (MP), a measure of semipermeability loss of the plasma membrane, was determined by assessing electrolyte leakage according to the method described by Valentovic et al. [36]. Five plants were randomly chosen per replicate and cut into uniformly sized discs; then, 0.5 g of the disc was taken from the middle portion of the youngest fully developed leaf, which was washed with distilled water to remove surface contamination. The discs were placed in closed tubes containing 20 mL of deionized water and incubated at 25 °C for 24 h. The electrical conductivity (EC) of the bathing solution (EC1) was determined. The samples were then boiled in a water bath at 120 °C for 20 min, and the electrical conductivity (EC2) was determined after cooling the solution to room temperature. Membrane permeability was calculated as a ratio as follows:

$$(\mathrm{EC1}/\mathrm{EC2}) \times 100$$

(C) The relative water content (RWC) estimates the water content of fresh sampled leaf tissue relative the maximal water content it can hold at full turgidity (condition of being swollen). It was measured in fully developed leaves, which were cut and weighed immediately to determine the fresh weight (FW) and then immersed in double-distilled water for 16 h. The excess surface water was removed by drying with paper towels to determine the turgid weight (TW) of the leaves. The leaves were then oven-dried until a constant weight was recorded as the dry weight (DW). The RWC was determined according to Anjum [37]:

The RWC was determined according to the following Equation:

$$\mathrm{RWC}\ (\%) = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

(D) The proline (an amino acid) content was determined according to the method developed by Bates et al. [38], which involves filtering 0.5 g fresh leaf material homogenized in 10 mL of 3% aqueous sulfosalicylic acid through Whitman's No. 1 filter paper. Then, 2 mL of filtered extract was taken for the analysis, and 2 mL acid ninhydrin and 2 mL glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h, and the reaction was finished in an ice bath. Then, 4 mL of toluene was added to the reaction mixture, and the organic phase was extracted and read at 520 nm by UV-visible spectrophotometer, using toluene as a blank.

(E) Ascorbate peroxidase (APX) was measured in plants according to [39] using a spectrophotometer (model UV—160A, Shimadzu, Japan).

(F) The chemical composition of samples of five fruits from each plot was determined 90 days after transplanting in both seasons by measuring the total soluble solids (TSS) and vitamin C (mg 100 g^{-1} FW) in fruit juice according to the methods described by the AOAC [40].

2.3.3. Flowering and Fruit Set Parameters

The number of flowers/plant, the number of fruits/plant, fruit set percentage, and number of seeds/fruit were recorded during the coldest period of the season in December and January to determine the most beneficial treatment for mitigating cold stress during this period.

2.3.4. Fruit Yield and Quality

Full-colored red fruits from the Zidenka hybrid and yellow fruits from the Lirica hybrid were selected to determine yield parameters. Early yield was defined as the number and weight of all harvested fruits during the first 45 days of picking. Data on the early and total yields include fruit yield per square meter (kg) and fruit number. Ten fruits were randomly taken from each plot to measure the average fruit length and diameter (cm), as well as the average fruit weight (g). Damaged and deformed fruits were considered unmarketable. All studied parameters of fruit yield and quality were measured at the Physiology and Breeding of Horticultural Crops Laboratory, Dept. of Horticulture, Fac. of Agric., Kafrelsheikh University, Kafr El-Sheikh, Egypt, an accredited lab according to the ISO/IEC 17025-2017 standard.

2.4. Soil Analysis

Soil samples were randomly collected at a depth of a 0–30 cm before conducting the experiments in both growing seasons. The soil texture was clayey, with pH values of 7.86 and 8.20 (in extract 1:2.5), 1.71 and 1.75 Mg/m^3 bulk density, 1.7 and 1.5% organic matter percentage, ESP 7.37% and 10.21%, water holding capacity of 37.3% and 36.7%, infiltration rate of 0.23 and 0.25 cm/h, 40.2% and 39.1% field capacity, and 3.94 and 4.38 dS m^{-1} EC (in extract 1:5) in the first and second season, respectively, indicating that the experimental soil had a high salinity level. Other values were previously determined in a soil and water suspension according to [41] at the Central Lab for Environmental Studies at Kafrelsheikh Uni., which is accredited under ISO/IEC 17025-2017.

2.5. Agricultural Practices

The seedlings were transplanted in soil in a Spanish-style unheated plastic greenhouse with 30 m width, 40 m length, and 3.5 m height on 10 November in the first season and October 15th in the second season. Two side ridges with a width of 1.5 m width and a length of 30 m were prepared with plant spacing of 50 cm. The plant density was 2.66 plants per m^2, and the experimental plot area was 6.25 m^2. Pruning was carried out on two branches 60 days after transplanting and continuously during the growing season. All missing plants were replaced within two weeks of transplanting. Common agricultural practices for drip irrigation, pest control, and fertilization were applied.

2.6. Statistical Analyses

Two factors in three replicates were studied in a split-plot design. The first factor was two pepper hybrids, which were assigned as main plots. The second factor included six treatments (chilling treatments with ozone levels of 0, 10, 20, 30, and 60 mg L^{-1}), which was assigned as a subplot. Statistical analysis was performed using the Assistats statistical software version 7.7 (2016). The main values of the treatments were compared according to Duncan's multiple range test at 0.05 levels for each season separately.

3. Results

3.1. Climatic and Microclimatic Conditions

Data presented in Figure 1 indicate that the air temperature in the studied greenhouse was higher than the outside temperature during all the experimentation periods from September until June. However, the temperature inside the greenhouse reached its minimum during January, with values of 7.8 and 7.5 °C in the first and second season, respectively. The minimum temperature also dropped to approximately 9–10 °C from November to February (about 120 days), indicating that the plants grown in the unheated greenhouse suffered from chilling stress for an extended period.

3.2. Relative Water Content of Leaves (RWC)

It is reasonable to follow up the changes in RWC in relation to the studied treatments because these changes have an impact on other important parameters, such as physiological characteristics, membrane permeability, enzyme activity, and solute concentrations. Overall, the RWC increased during the later sampling dates (60 and 90 days after transplanting (DAT)) compared with younger plant ages (Table 1). On all sampling dates during both growing seasons, the RWC was significantly affected by chilling tolerance pretreatments, with minor variations between the studied hybrids. Therefore, increasing the ozone concentration up to 20 or 30 ppm (in the first and second season, respectively) significantly increased the RWC in leaves. In comparison with cold pretreatment, it was higher than the untreated control, with intermediate values. With respect to the ozone application method, we did not find any significant differences between any sampling dates in either season, except after 60 DAT in the second season, for which the soaking method caused a much higher RWC than ozone seedling spraying.

Table 1. Effect of ozone and chilling tolerance pretreatments on relative water content of two sweet pepper hybrids 30, 60, and 90 days after transplanting during the 2017/18 and 2018/19 seasons.

Treatment		Relative Water Content (%)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018							
Zidenka cv.	Control	72.43 d	71.50 f	81.10 c	79.46 e	81.00 b	81.13 b
	* Cold	75.46 b	76.13 b	82.06 b	81.90 bc	81.20 b	81.30 b
	1 ppm O_3	72.60 d	71.40 f	81.16 c	79.40 e	81.30 b	81.20 b
	5 ppm O_3	72.10 d	71.50 f	81.23 c	79.56 e	81.50 b	81.40 b
	10 ppm O_3	74.26 c	73.06 e	81.56 c	81.66 c	81.40 b	81.50 b
	20 ppm O_3	75.20 b	75.70 bc	82.30 b	82.03 b	82.06 a	82.16 a
Lirica cv.	Control	74.30 c	74.40 d	82.00 bc	81.00 c	81.06 b	80.96 ab
	* Cold	74.26 c	76.13 b	82.56 b	81.33 c	81.06 b	81.26 b
	1 ppm O_3	74.06 c	74.36 d	82.00 bc	81.10 c	81.26 b	81.06 bc
	5 ppm O_3	74.30 c	74.50 d	82.03 b	81.43 c	81.80 ab	81.73 b
	10 ppm O_3	75.46 b	75.06 c	84.43 a	84.20 a	81.73 ab	81.80 b
	20 ppm O_3	78.63 a	78.33 a	85.23 a	85.13 a	82.06 a	82.33 a
Significance		*	*	*	*	*	*
2018/2019							
Zidenka cv.	Control	71.20 i	71.50 g	79.60 e	79.63 f	81.00 cd	81.13 c
	* Cold	76.60 e	76.13 d	81.90 d	81.20 c	81.20 c	81.30 c
	10 ppm O_3	73.33 g	73.06 f	82.00 cd	79.86 f	81.40 c	81.50 c
	20 ppm O_3	76.23 e	75.70 e	83.26 c	80.03 e	82.06 b	82.16 b
	30 ppm O_3	81.36 b	81.00 b	89.73 a	81.93 bc	83.00 a	83.13 ab
	60 ppm O_3	77.20 d	81.13 b	84.63 b	82.56 b	82.93 b	83.23 a

Table 1. *Cont.*

Treatment		Relative Water Content (%)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2018/2019							
Lirica cv.	Control	74.30 fg	74.40 ef	80.86 e	79.83 f	81.06 cd	80.96 d
	* Cold	76.70 e	76.50 d	82.66 c	81.16 c	81.06 cd	81.26 c
	10 ppm O_3	75.46 f	75.06 e	84.53 b	80.70 e	81.73 c	81.80 c
	20 ppm O_3	78.63 cd	78.23 c	85.20 b	81.36 c	82.06 b	82.33 b
	30 ppm O_3	83.83 a	82.90 a	91.76 a	83.70 ab	84.06 a	84.40 a
	60 ppm O_3	79.30 c	82.20 a	84.66 b	84. 93 a	83.60 a	83.23 a
Significance		*	*	*	*	*	*

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

3.3. Plant Leaf Area

The results presented in Table 2 show that the plant leaf area almost doubled from 30 to 60 (DAT). The interaction between the hybrids and chilling tolerant treatments was significant in both growing seasons, except in the first season on the early sampling date (30 DAT). It was observed that regardless the hybrid, the untreated control and cold pretreatment obtained the lower values than ozone pretreatment. However, the higher ozone concentrations either with seed soaking or seedling spray significantly increased plant leaf area. Ozone at 20 ppm produced the highest plant leaf area in the first season, followed by 10 ppm, whereas in the second season, 30 ppm of ozone achieved the highest values. Moreover, data show insignificant differences between the application methods of seed soaking and seedling spray in terms of plant leaf area, except at 60 DAT in the first season, when seedling spray had a better effect than seed soaking. However, the plant leaf area of the Lirica's hybrid was somewhat greater than that of the Zidenka hybrid, with maximum values recorded when exposed to ozone levels of 20 or 30 ppm.

Table 2. Effect of ozone and chilling tolerance pretreatments on plant leaf area of two sweet pepper hybrids 30 and 60 days after transplanting during the 2017/18 and 2018/19 seasons.

Treatment		Leaf Area (m^2 $Plant^{-1}$)			
		30 Days after Transplanting		60 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018					
Zidenka cv.	Control	15.68 a	15.47 a	30.01 e	31.09 d
	* Cold	15.00 a	15.00 a	33.62 b	31.40 d
	1 ppm O_3	15.88 a	15.31 a	30.56 e	31.58 d
	5 ppm O_3	15.88 a	16.03 a	30.64 e	31.25 d
	10 ppm O_3	16.86 a	17.01 a	32.43 c	34.15 bc
	20 ppm O_3	17.51 a	17.29 a	36.58 a	35.73 b
Lirica cv.	Control	15.90 a	15.27 a	31.09 d	33.57 c
	* Cold	15.34 a	15.42 a	31.40 d	35.72 b
	1 ppm O_3	16.19 a	16.00 a	31.58 d	33.31 c
	5 ppm O_3	16.26 a	17.32 a	31.25 d	33.62 c
	10 ppm O_3	17.03 a	17.37 a	34.15 b	40.39 a
	20 ppm O_3	17.95 a	15.84 a	35.75 a	36.37 b
Significance		ns	ns	*	*

Table 2. *Cont.*

Treatment		Leaf Area (m^2 $Plant^{-1}$)			
		30 Days after Transplanting		60 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2018/2019					
Zidenka cv.	Control	18.50 cd	17.55 d	26.12 h	28.77 f
	* Cold	15.92 e	18.07 cd	31.55 g	42.42 c
	10 ppm O_3	19.13 c	19.49 b	34.00 f	38.26 d
	20 ppm O_3	19.77 bc	19.64 b	41.32 d	44.20 b
	30 ppm O_3	21.59 a	19.28 b	43.96 c	47.03 a
	60 ppm O_3	19.92 bc	18.77 c	45.04 b	34.09 e
Lirica cv.	Control	16.85 d	16.71 e	25.73 h	26.84 g
	* Cold	15.98 de	15.89 e	30.38 g	27.80 f
	10 ppm O_3	20.18 b	19.13 bc	37.55 e	33.34 e
	20 ppm O_3	20.96 ab	21.94 a	43.91 c	37.75 d
	30 ppm O_3	21.65 a	20.83 ab	49.36 a	48.33 a
	60 ppm O_3	21.05 a	18.20 c	44.93 b	38.84 d
Significance		*	*	*	*

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

3.4. Relative Chlorophyll Content of Leaves (SPAD)

The relative chlorophyll content (SPAD) seemed to rise with plant age until 90 DAT (Table 3). Lirica leaves tended to have the highest relative chlorophyll content when exposed to high ozone concentrations (20 and 30 ppm). Increasing the ozone concentration to 20 ppm (in the first season) or 30 ppm (in the second season) significantly increased SPAD values during the first three months of plant life. However, the control and cold pretreatment resulted in the lowest values. Except at early sampling dates (30 and 60 DAT) in the second season, the differences between the two application methods (seed soaking or seedling spray) were not statistically detectable, and seed soaking was superior to seedling spray. In both growing seasons at all sampling dates, the interaction between the hybrids and chilling tolerance pretreatments was significant (Table 2).

Table 3. Effect of ozone and chilling tolerance pretreatments on relative chlorophyll content (SPAD) of two sweet pepper hybrids 30, 60, and 90 days after transplanting during the 2017/18 and 2018/19 seasons.

Treatment		Relative Chlorophyll Content (SPAD)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018							
Zidenka cv.	Control	43.06 e	41.43 e	49.46 e	49.30 e	58.30 c	58.46 b
	* Cold	49.10 c	46.60 c	52.20 c	54.60 bc	58.33 c	58.13 bc
	1 ppm O_3	42.56 ef	41.50 e	49.63 e	49.36 e	58.13 c	58.33 b
	5 ppm O_3	43.86 e	41.30 e	49.76 e	49.40 e	58.13 c	58.13 bc
	10 ppm O_3	48.13 d	43.46 d	54.10 b	51.46 d	58.13 c	58.13 bc
	20 ppm O_3	53.20 ab	47.13 bc	54.83 b	55.13 b	59.10 b	59.10 ab
Lirica cv.	Control	48.50 cd	48.13 b	52.06 c	53.90 c	58.80 bc	58.50 b
	* Cold	48.03 d	47.60 bc	52.60 c	55.60 b	59.70 ab	59.70 b
	1 ppm O_3	48.50 cd	48.30 b	52.33 c	55.13 b	59.70 ab	59.70 a
	5 ppm O_3	48.40 cd	48.60 b	52.36 c	56.40 a	60.13 a	60.13 a
	10 ppm O_3	52.73 b	49.50 a	54.70 b	56.13 a	60.13 a	60.13 a
	20 ppm O_3	54.76 a	50.13 a	57.40 a	56.20 a	60.13 a	61.06 a

Table 3. *Cont.*

Treatment		Relative Chlorophyll Content (SPAD)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
Significance		*	*	*	*	*	*
2018/2019							
Zidenka cv.	Control	41.20 e	42.30 e	51.20 g	51.30 e	58.30 c	58.46 c
	* Cold	41.80 e	46.60 c	51.80 g	56.60 c	58.33 c	58.13 c
	10 ppm O_3	48.13 d	43.66 d	58.13 e	53.46 d	58.13 c	58.13 c
	20 ppm O_3	49.10 c	47.13 b	59.10 d	57.13 b	59.10 b	59.10 bc
	30 ppm O_3	53.13 ab	48.76 a	63.13 b	58.76 a	60.13 a	60.13 ab
	60 ppm O_3	48.20 d	45.66 cd	58.20 e	55.66 c	59.76 ab	59.23 bc
Lirica cv.	Control	48.50 cd	48.13 ab	58.50 de	58.13 ab	58.80 c	58.80 c
	* Cold	44.60 e	47.60 ab	54.60 f	57.60 b	59.70 ab	59.70 b
	10 ppm O_3	52.73 b	45.90 cd	62.73 bc	55.90 c	60.13 a	60.13 ab
	20 ppm O_3	53.20 ab	47.13 b	63.20 b	57.13 b	60.13 a	61.06 a
	30 ppm O_3	54.76 a	49.10 a	64.76 a	59.10 a	60.36 a	60.36 a
	60 ppm O_3	52.13 b	48.56 a	62.13 c	58.56 a	60.00 a	59.83 b
Significance		*	*	*	*	*	*

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

3.5. Membrane Permeability

The data presented in Table 4 indicate that the membranes were more efficient during early plant growth (30 DAT), and their effectiveness then declined. In general, at all sampling dates during both growing seasons, the studied pretreatments had a significant impact on the permeability of the leaf cell membrane, and slight differences between the application methods (seed soaking and seedling spray) were noticed. The treatments increased the membrane stability compared to the untreated control. However, the results with respect to the studied hybrids are in conflict. In the first season at early sampling dates, the control did not significantly differ from the plants treated with low ozone concentrations. Later, at 90 DAT, all ozone concentrations were significantly higher than the untreated control. Regardless of the treated hybrid, plants treated with higher ozone concentrations (20 ppm in the first season and 30 and 60 ppm in the second season) had the highest values, whereas the cold pretreatment had intermediate values.

Table 4. Effect of ozone and chilling tolerance pretreatments on membrane permeability of two sweet pepper hybrids 30, 60, and 90 days after transplanting during the 2017/18 and 2018/19 seasons.

Treatment		Membrane Permeability (%)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018							
Zidenka cv.	Control	18.23 a	18.23 a	22.13 a	22.56 a	21.06 a	21.20 a
	* Cold	16.36 c	16.36 c	19.63 c	19.70 c	19.96 b	20.66 ab
	1 ppm O_3	18.30 a	18.23 a	21.50 b	22.53 a	17.13 e	16.66 cd
	5 ppm O_3	18.03 a	18.36 a	21.76 ab	22.60 a	17.96 e	16.43 d
	10 ppm O_3	17.23 b	17.23 b	21.76 ab	21.53 b	19.66 b	19.50 b
	20 ppm O_3	15.20 d	15.20 d	26.60 e	21.40 b	19.03 b	18.80 bc

Table 4. *Cont.*

Treatment		Membrane Permeability (%)					
		30 Days after Transplanting		60 Days after Transplanting		90 Days after Transplanting	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
Lirica cv.	Control	17.93 ab	17.93 ab	20.32 bc	19.73 c	18.60 c	19.73 b
	* Cold	16.56 c	16.56 c	18.90 cd	18.66 d	18.50 c	18.60 bc
	1 ppm O_3	18.13 a	17.56 ab	21.06 b	19.70 c	18.26 c	18.13 c
	5 ppm O_3	18.26 a	17.86 ab	20.53 bc	19.60 c	17.40 e	17.23 c
	10 ppm O_3	17.40 b	17.40 b	17.73 d	17.93 de	18.03 c	18.03 c
	20 ppm O_3	15.66 d	15.66 d	17.00 d	17.13 e	17.60 e	17.60 cd
Significance		*	*	*	*	*	*
2018/2019							
Zidenka cv.	Control	18.23 a	18.23 a	22.13 a	22.56 a	21.06 a	21.20 a
	* Cold	16.36 c	16.36 c	19.63 d	19.70 c	19.96 ab	20.06 b
	10 ppm O_3	17.23 b	17.23 b	21.50 b	22.53 a	19.66 b	19.50 b
	20 ppm O_3	15.20 d	15.20 d	21.76 ab	22.60 a	19.03 b	18.80 bc
	30 ppm O_3	14.46 e	14.46 e	21.70 ab	21.53 b	18.26 c	18.13 c
	60 ppm O_3	16.30 c	14.96 d	20.60 c	21.40 b	17.40 d	17.23 cd
Lirica cv.	Control	17.93 a	17.93 a	20.23 c	22.53 a	18.60 bc	19.73 b
	* Cold	16.56 c	16.56 c	18.90 de	18.66 e	18.50 bc	18.60 bc
	10 ppm O_3	17.40 b	17.40 ab	21.06 b	19.73 c	18.03 c	18.03 c
	20 ppm O_3	15.66 d	15.66 d	20.53 c	19.60 c	17.60 d	17.60 c
	30 ppm O_3	14.06 e	14.06 e	17.73 e	17.93 f	17.13 d	16.66 d
	60 ppm O_3	17.23 b	14.80 e	17.00 e	17.13 e	17.96 cd	16.43 d
Significance		*	*	*	*	*	*

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

3.6. Proline Content of Leaves

At 60 DAT, proline content jumped to almost three times more than that at 30 DAT, then slightly decreased at 90 DAT (Figures 2 and 3). The proline content in Lirica leaves was significantly higher than that in Zidenka leaves. Lirica leaves had the highest proline content under 20 or 30 ppm ozone in the first and second season, respectively. Chilling tolerance pretreatment significantly affected leaf proline content at all sampling dates (30, 60, and 90 DAT) in both seasons. Proline content increased gradually as O_3 concentration was increased to 30 or 60 ppm. At all tested sampling dates in both seasons, cold pretreatment ranked second, whereas the control recorded the lowest value. Seedling spray was better than seed soaking at 30 DAT, increased from 0.806 to 1.645 and from 0.874 to 0.993 μmol g^{-1}FW in the first and second season, respectively, with increases from 0.911 to 1.75 and from 1.761 to 1.874 μmol g^{-1} in the first and second season, respectively, at 60 DAT, whereas the differences were not statistically detectable at 90 DAT.

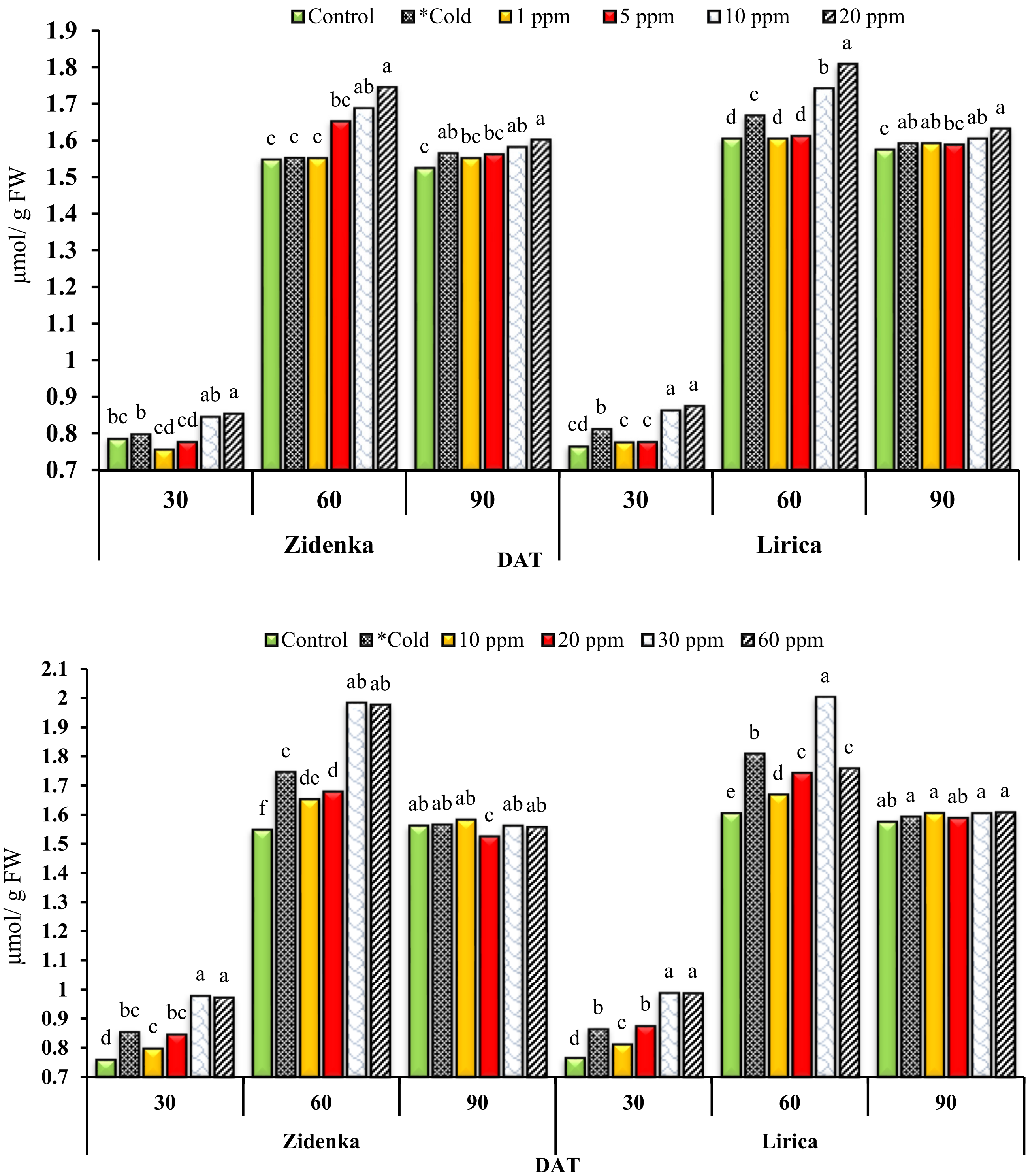


Figure 2. Effect of seed soaking with different ozonated water concentrations and cold pretreatments on the proline content of two pepper hybrids 30, 60, and 90 days after transplanting during the 2017/2018 (**upper** figure) and 2018/2019 (**lower** figure) seasons. Columns labeled with a lowercase letter are not significantly different according to Duncan's multiple range test at 0.05 levels for the same sampling date in each season and each hybrid separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water was applied as seed soaking. The average value of three biological and three technical replicates is reported.

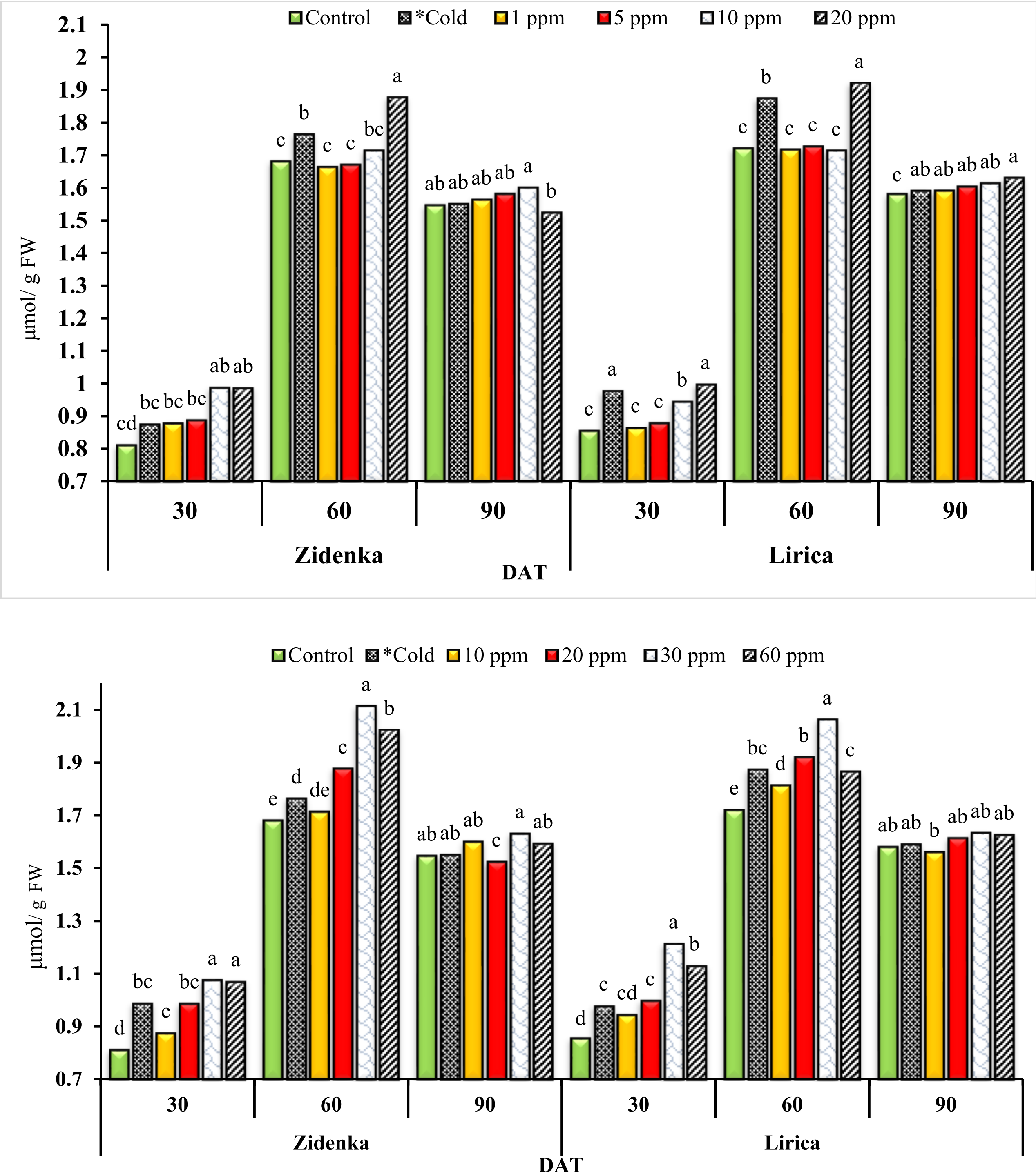


Figure 3. Effect of seedlings spray with different ozonated water concentrations and cold pretreatments on proline content of two pepper hybrids 30, 60, and 90 days after transplanting during the 2017/2018 (**upper** figure) and 2018/2019 (**lower** figure) seasons. The columns labeled with a lowercase letter are not significantly different according to Duncan's multiple range test at 0.05 levels for the same sampling date in each season and each hybrid separately. * Cold stress treatment was performed at 4 °C for 36 h. The average value of three biological and three technical replicates is reported.

3.7. Ascorbate Peroxidase (APX)

According to the data shown in Figures 4 and 5, APX almost doubled 60 days after transplanting compared to after 30 days, then slightly decreased after 90 days. Data indicate that ozone and cold pretreatments had a significant impact on APX activity in both hybrid leaves at all tested sample dates during both growing seasons. In general, Lirica leaves had the highest APX activity when treated with higher ozone-induced concentrations. However, the Zidenka control had the lowest values. APX activity increased with increasing ozone concentration and reached the maximum value in Lyrica leaves with 20 or 30 ppm O_3 (in the first and second season, respectively). The cold treatment resulted in intermediate values between the higher concentrations of ozone and the control. With respect to the application method, it was found that APX activity was significantly higher when seedlings were sprayed by ozone than when the seeds were soaked in ozonated water at a young plant age 30 and 60 days after transplanting. The enzyme activity increased from 0.333–0.362 to 0.378–0.412 μmol $min^{-1}g$ at 30 DAT and from 0.683–0.731 to 0.731–0.778 μmol $min^{-1}g$ at 60 DAT in the first and second season, respectively. However, the differences were not statistically detectable at 90 DAT in either growing season.

3.8. Flowering and Fruit Set Parameters

To investigate the efficiency of treatments in mitigating cold stress, flowering and fruit set parameters were monitored during the coldest period of the year in December and January (Table 5). Among all examined flowering and fruit set characteristics, the interaction between the hybrids and prime treatments was significant. Additionally, a considerable increase in pollination and fertilization over the control and cold treatment was suggested. It was noticed that Lirica hybrid's flowering was extremely sensitive to cold treatment, as reflected by the poor flower production of the plant (3.66–4.20 flower/plant) during the coldest period of December and January. However, it produced the most flowers (6.66 to 7.33 flowers/plant) when primed by high ozone concentrations. Furthermore, when exposed to increased ozone concentrations, Larica had a higher fruit set percentage, more fruits per plant, and more seeds inside each fruit (Table 5). Ozone pretreatment had a superior effect in terms of pollination and fertilization processes under cold stress, as evidenced by the considerable increases in the number of flowers per plant (from 4.76–5.20 to 6.33–7.33 flowers) and fruit set percentage (from 34.86–45.50 to 93.19–99.24%), which resulted from increasing the ozone concentration up to 20 ppm in the first season and 30 ppm in the second season. It was apparent that the trend in the number of fruits formed by each plant followed that of flowers. Cold treatment and the control had the lowest values in both seasons. As an indicator of fertilization efficacy, we counted the seeds inside the fruit. When the seeds and seedlings were previously primed by ozonated water with concentrations of 20 and 30 ppm, the fruits had high seed counts. The control and the cold treatment plants recorded had the lowest values in both seasons. Regarding application techniques, we found no significant variations in any of the blooming and fruit set features investigated in either growing season.

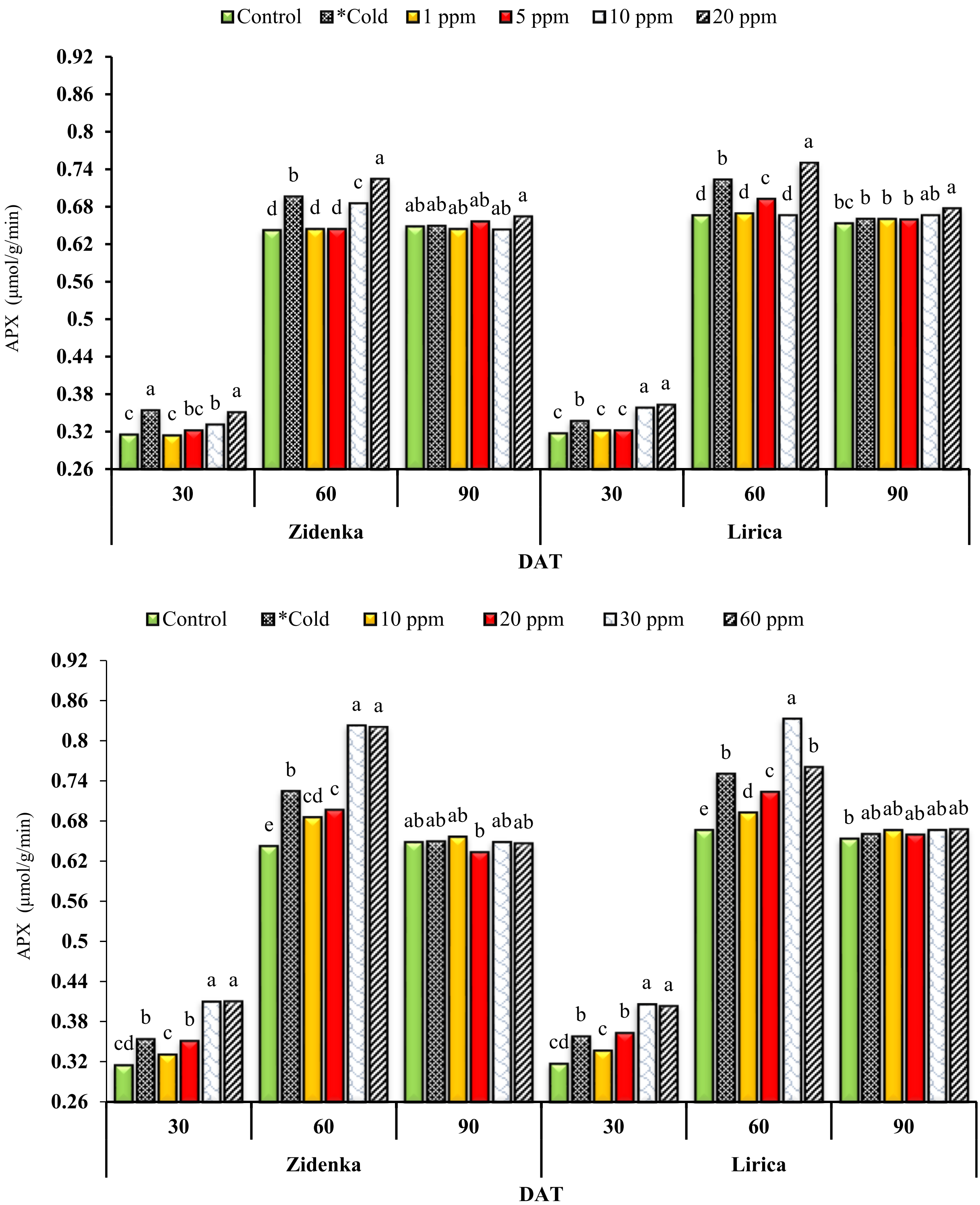


Figure 4. Effect of seed soaking in different ozonated water concentrations and cold pretreatments on APX activity of two pepper hybrids 30, 60, and 90 days after transplanting during the 2017/2018 (**upper** figure) and 2018/19 (**lower** figure) seasons. The column followed by a lowercase letter are not significantly different according to Duncan's multiple range test at 0.05 levels for the same sampling date of each season and each hybrid separately. * cold treatment was performed at 4 °C for 36 h. The average value of three biological and three technical replicates is reported.

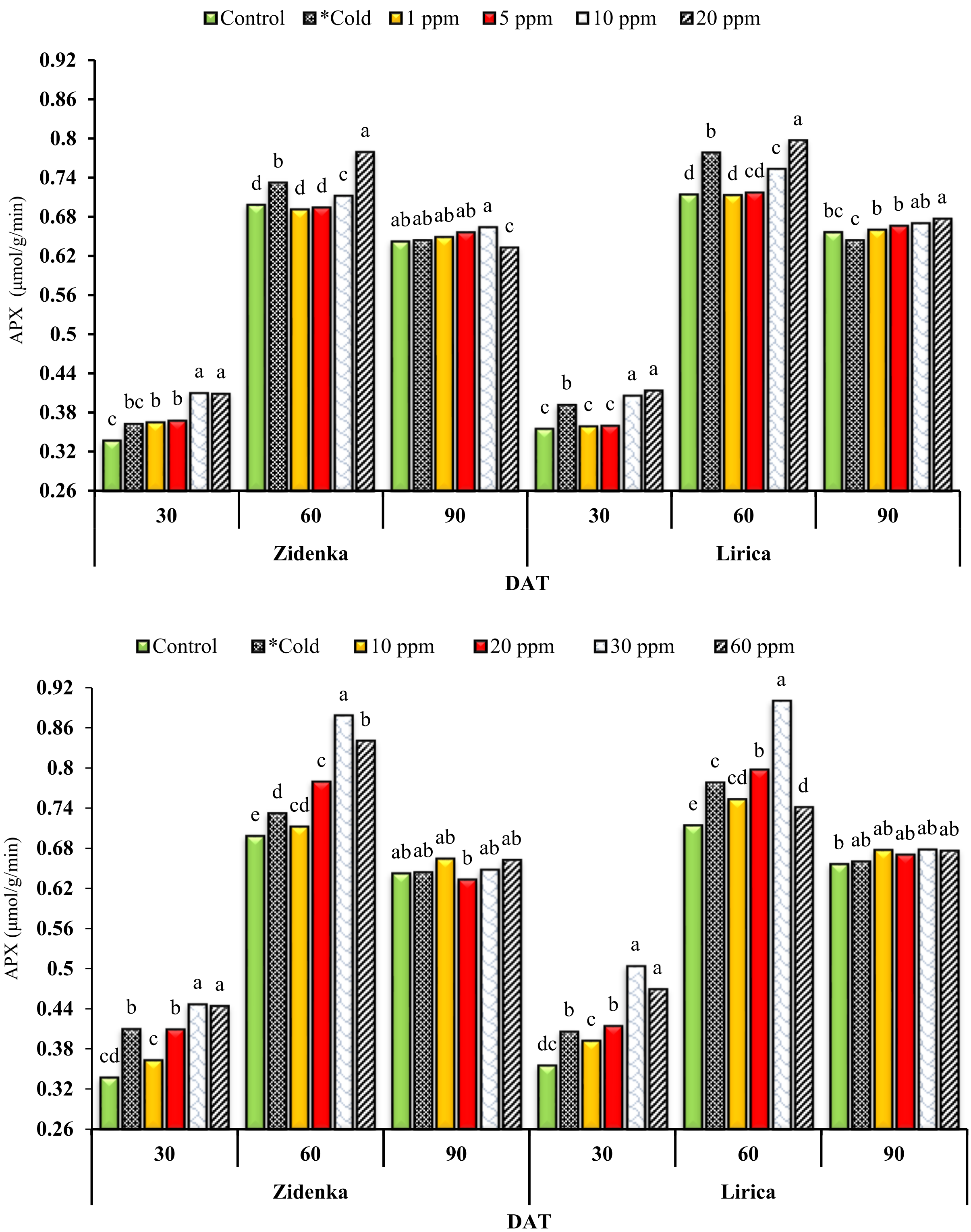


Figure 5. Effect of seedlings spray with different ozonated water concentrations and cold pretreatments on APX activity of two pepper hybrids 30, 60, and 90 days after transplanting during the 2017/2018 (**upper** figure) and 2018/2019 (**lower** figure) seasons. Columns labeled with a lowercase letter are not significantly different according to Duncan's multiple range test at 0.05 levels for the same sampling date in each season and each hybrid separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seedling spray compared to the untreated control. The average value of three biological and three technical replicates is reported.

Table 5. Effect of ozone and chilling tolerance pretreatments on flowering and fruit set parameters of two sweet pepper hybrids during December and January of the 2017/18 and 2018/19 seasons.

Treatment		No. Flowers/Plant		No. Fruits/Plant		No. Seeds/Fruit		Fruits Set %	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018									
Zidenka cv.	Control	5.00 c	5.00 bc	1.66 e	1.66 d	261 e	261 d	38.77 e	34.86 f
	* Cold	4.66 cd	4.66 c	3.00 c	3.00 bcd	263 e	263 d	65.00 c	65.00 d
	1 ppm O_3	4.33 d	5.33 b	2.66 cd	3.66 bc	307 cd	254 d	65.41 c	69.44 c
	5 ppm O_3	5.33 bc	5.00 bc	2.33 d	3.00 bcd	290 d	277 cd	55.16 d	69.11 c
	10 ppm O_3	4.66 cd	5.00 bc	3.00 c	3.66 bc	315 c	303 bc	56.00 d	93.65 a
	20 ppm O_3	6.00 ab	5.66 b	4.33 ab	5.33 a	322 b	339 c	73.40 b	88.29 b
Lirica cv.	Control	4.66 cd	4.66 c	3.00 c	3.33 bc	280 d	280 cd	63.44 bc	79.65 b
	* Cold	3.66 e	3.66 d	2.33 d	2.33 cd	313 c	313 c	66.60 b	68.33 c
	1 ppm O_3	5.66 b	5.00 bc	4.33 ab	2.33 cd	311 c	360 b	78.66 b	50.62 e
	5 ppm O_3	5.33 bc	5.00 bc	5.66 a	4.66 b	314 c	356 b	57.93 d	83.45 b
	10 ppm O_3	6.33 ab	5.66 b	3.66 b	5.33 a	341 ab	412 a	77.98 b	91.62 ab
	20 ppm O_3	7.00 a	7.33 a	5.66 a	6.00 a	364 a	403 a	99.16 a	97.77 a
Significance		*	*	*	*	*	*	*	*
2018/2019									
Zidenka cv.	Control	5.20 bc	4.76 bc	2.06 d	2.16 de	283 d	283 d	40.47 e	45.50 e
	* Cold	4.86 c	4.93 b	2.96 cd	3.13 cd	276 d	276 de	60.83 cd	63.82 c
	10 ppm O_3	4.53 cd	5.53 ab	2.66 d	3.23 cd	322 c	268 e	59.37 cd	59.55 d
	20 ppm O_3	5.26 bc	5.43 ab	2.66 d	3.13 cd	304 c	324 c	52.45 d	59.56 d
	30 ppm O_3	5.93 ab	6.50 a	4.60 d	4.86 b	348 b	355 bc	78.28 b	75.30 b
	60 ppm O_3	5.93 ab	5.80 a	4.53 b	5.53 ab	329 b	318 c	74.08 bc	92.13 a
Lirica cv.	Control	5.03 c	5.20 ab	3.33 c	3.46 bc	294 d	294 d	65.99 c	73.13 bc
	* Cold	4.20 d	4.20 c	2.66 d	2.73 d	328 bc	328 c	64.42 c	63.97 c
	10 ppm O_3	5.53 b	4.86 b	4.53 b	2.66 d	327 bc	374 b	79.69 b	54.80 d
	20 ppm O_3	5.20 bc	5.53 ab	5.83 a	4.86 b	329 b	376 b	81.92 b	88.21 ab
	30 ppm O_3	7.26 a	6.66 a	5.80 a	6.16 a	382 a	423 a	99.24 a	93.19 a
	60 ppm O_3	6.66 a	6.33 a	4.06 bc	5.76 ab	358 a	421 a	61.26 c	91.50 a
Significance		*	*	*	*	*	*	*	*

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

3.9. Fruit Yield and Its Components

Full-colored red fruits from the Zidenka hybrid and yellow fruits from the Lirica hybrid were ready for picking on 20 February in the first season and 10 February in the second season, and picking continued until July in both seasons. The studied yield parameters were significantly affected by the interaction between hybrids and priming treatments (Table 6). In comparison with the control and cold treatment, ozone-induced pretreatment of Lirica seeds or seedlings, particularly at concentrations of 20 and 30 ppm in the first and second seasons, provided the highest early yield with the highest average fruit weight and fruit numbers. However, the control and cold treatment had the lowest values in both seasons. The early yield increased from 4.95 kg m^{-2} (control average) to 7.34 kg m^{-2} (average with 20 and 30 ppm ozone). Lirica provided the maximum early yield when primed by ozone at 30 ppm (9.87 kg m^{-2}), as well as the maximum fruits per m^2 (29.19 fruit), and average fruit weight (234 g). In contrast, the lowest obtained values were obtained in the Lirica control (4.56 kg m^{-2} early yield and 16.81 fruits m^{-2}), whereas the Zidenka control produced the lowest average fruit weight (164 g). We did not detect any significant changes between plants treated with ozone by either foliar spray or seed soaking (Table 6).

Table 6. Effect of ozone and chilling tolerance pretreatments on fruit yield parameters of two sweet pepper hybrids during the 2017/18 and 2018/19 seasons.

Treatment		[+] Early Yield (kg m^{-2})		Fruits per m^{-2} for Early Yield		Average Fruit Weight (g) for Total Yield	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018							
Zidenka cv.	Control	4.76 d	5.75 bc	24.44 ab	26.41 ab	164 cd	172 cd
	* Cold	4.80 d	5.49 c	24.53 ab	21.60 c	175 c	173 cd
	1 ppm O_3	4.63 d	4.52 c	19.75 d	25.82 b	177 c	172 cd
	5 ppm O_3	5.04 c	4.88 d	24.83 b	21.98 cd	169 c	186 c
	10 ppm O_3	5.05 c	5.23 c	23.60 bc	23.84 c	179 c	169 d
	20 ppm O_3	5.89 bc	5.14 cd	26.44 a	23.55 c	175 c	171 cd
Lirica cv.	Control	4.36 e	4.80 d	16.81 e	19.75 d	220 a	205 b
	* Cold	4.57 de	5.50 c	17.60 e	22.03 cd	212 a	206 b
	1 ppm O_3	5.24 c	5.90 bc	21.27 d	20.99 d	200 b	206 b
	5 ppm O_3	6.04 b	5.36 c	23.26 bc	19.56 de	209 ab	207 b
	10 ppm O_3	6.11 b	6.69 b	22.63 c	23.35 c	206 ab	218 a
	20 ppm O_3	7.71 a	8.98 a	25.37 a	28.24 a	222 a	229 a
Significance		*	*	*	*	*	*
2018/2019							
Zidenka cv.	Control	5.11 c	5.64 d	25.55 b	25.39 b	173 c	175 d
	* Cold	5.14 c	5.61 d	25.36 b	26.70 ab	184 c	180 cd
	10 ppm O_3	5.04 c	4.92 e	20.43 e	22.34 c	183 c	176 d
	20 ppm O_3	5.49 cd	5.32 d	25.67 b	22.73 c	178 c	196 c
	30 ppm O_3	6.61 b	6.08 c	27.91 a	26.30 ab	184 c	179 d
	60 ppm O_3	5.01 cd	5.38 d	22.23 d	23.20 c	187 c	181 d
Lirica cv.	Control	4.56 d	4.59 e	17.72 e	18.99 de	230 a	212 b
	* Cold	4.97 cd	5.93 cd	17.38 f	22.78 c	223 ab	215 b
	10 ppm O_3	5.71 bc	6.42 c	21.99 de	21.70 d	211 b	213 b
	20 ppm O_3	6.58 b	4.98 e	24.05 c	17.33 e	220 ab	218 b
	30 ppm O_3	8.49 a	9.87 a	26.23 ab	29.19 a	234 a	241 a
	60 ppm O_3	6.65 b	7.28 b	23.39 cd	24.14 b	203 b	230 a
Significance		*	*	*	*	*	*

[+] The early yield was determined during the first 45 days of picking. Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

During both growing seasons, the total and marketable yields of the examined hybrids significantly responded to pretreatments for chilling tolerance; however, the nonmarketable yield did not exhibit any significant response (Figures 6 and 7). All ozone pretreatments resulted in a significant increase in total and marketable fruit yield compared with the control and cold treatment in both seasons. Lirica had higher total and marketable yields than Zidenka. The total yield increased from 11.57–12.29 to 14.76–16.13 kg/m^2 when Lirica was treated by 20 ppm in the first season and from 12.64–13.36 to 16.04–16.52 kg/m^2 in the second season, with an increased in the marketable yield from 11.21–11.83 to 14.33–15.56 and from 12.24–12.85 to 15.43–16.10 kg/m^2, in the first and second seasons, respectively. The total and marketable yields of Zidenka were lowest, especially in the control and the plant treated with slow ozone concentration. Therefore, there were non-significant differences between the application methods, i.e., seed soaking and seedling spray.

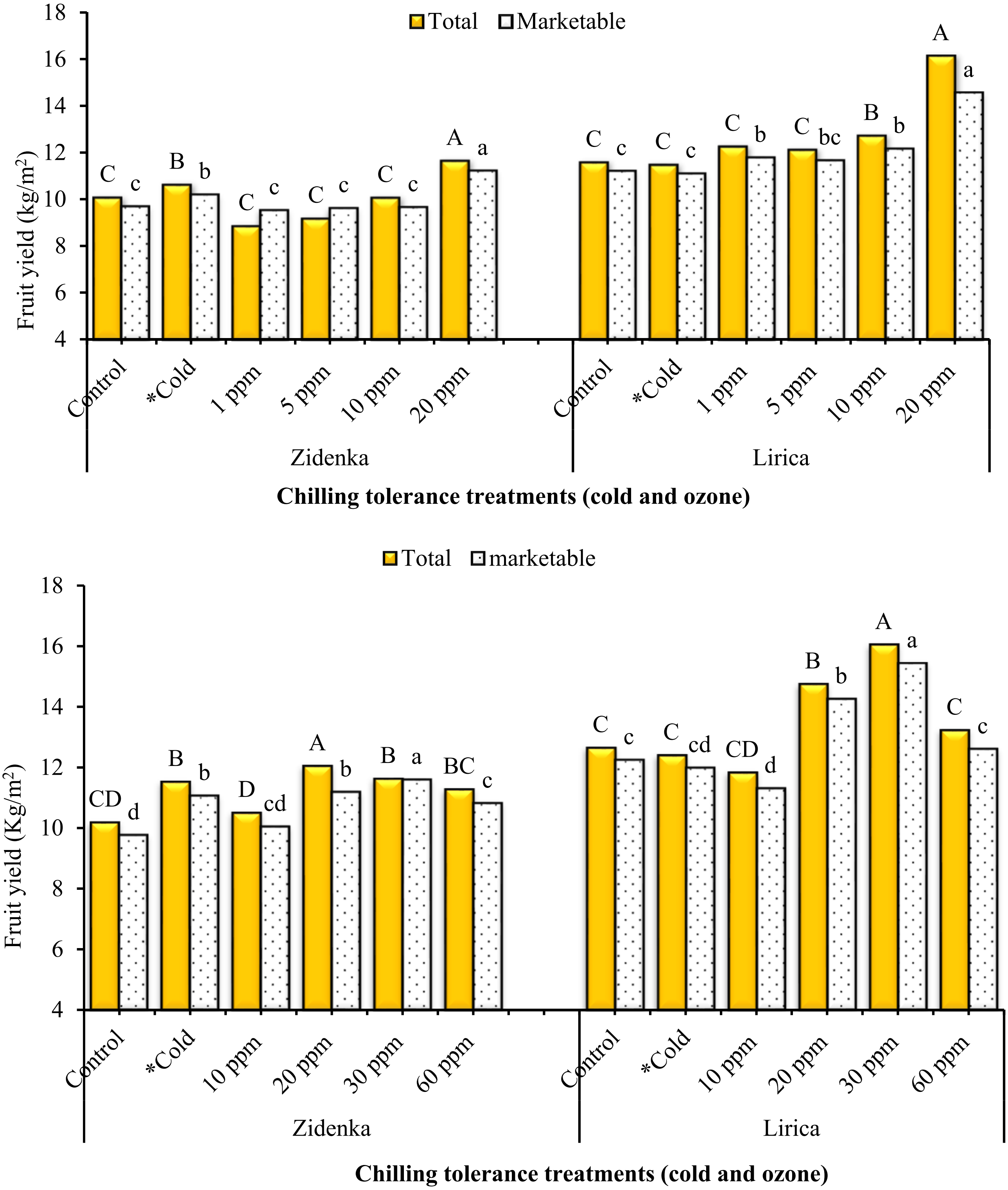
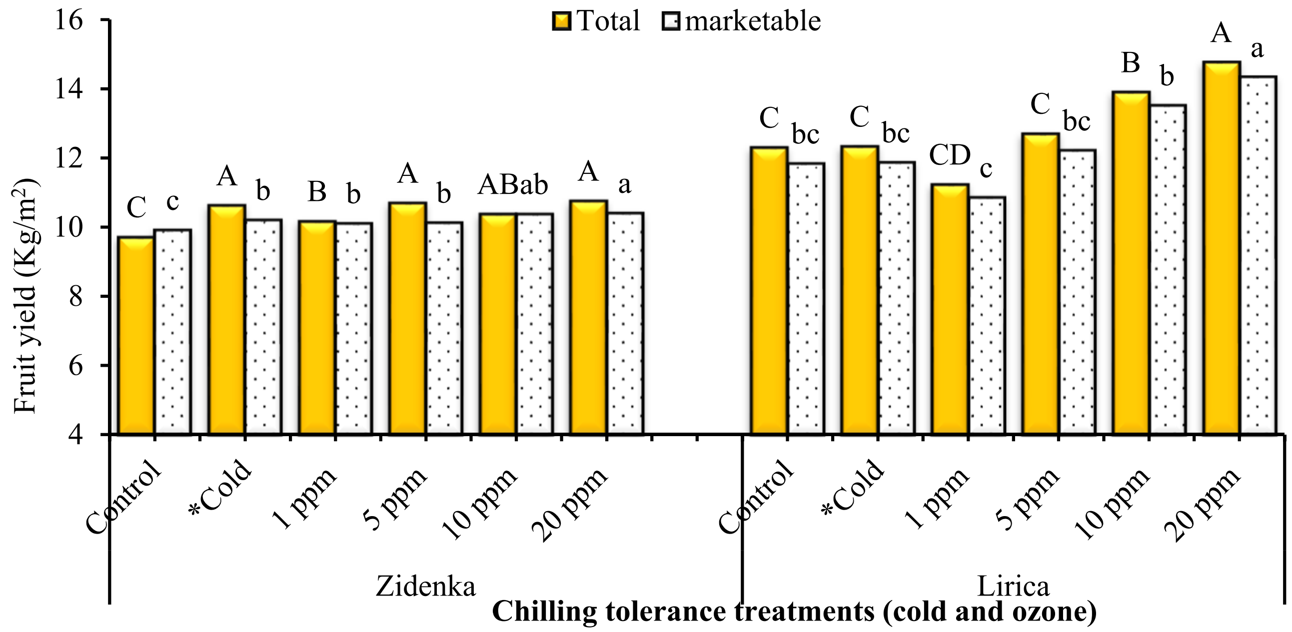


Figure 6. Effect of seed soaking in different ozonated water concentrations and cold pretreatments on total and marketable fruit yields of two sweet pepper hybrids during the 2017/2018 (**upper** figure) and 2018/2019 (**lower** figure) seasons. The columns labeled with different letters with in the same case are significantly different according to Duncan's multiple range test at 0.05 levels. * Cold stress treatment was performed at 4 °C for 36 h.



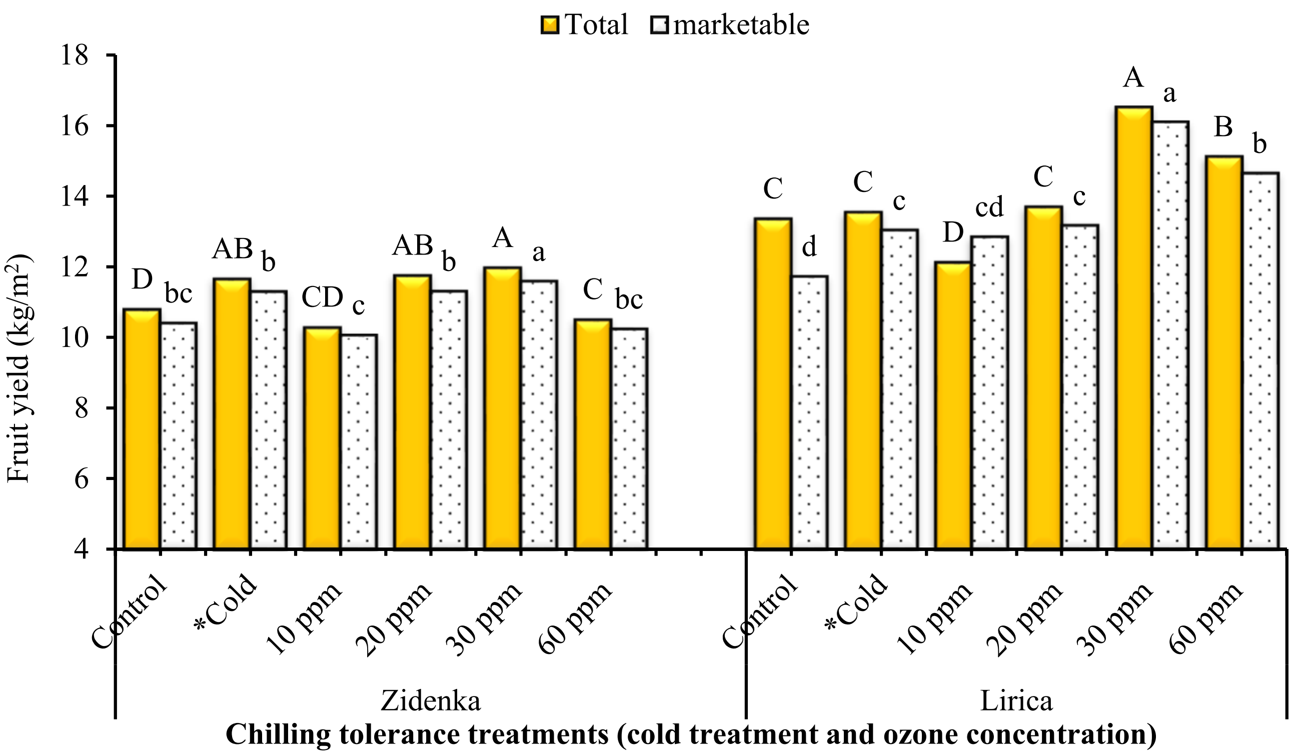


Figure 7. Effect of seedlings spray with different ozonated water concentrations and cold pretreatments on total and marketable fruit yields of two sweet pepper hybrids during the 2017/2018 (**upper** figure) and 2018/2019 (**lower** figure) seasons. The columns labeled with different letters in the same case are significantly different according to Duncan's multiple range test at 0.05 levels. * Cold treatment was performed at 4 °C for 36 h.

3.10. Fruit Quality

The results presented in Table 7 indicate that increasing ozone concentrations significantly increased fruit length of both hybrids during both seasons. The fruit of the Lirica hybrid longest, increasing from 8.46–9.83 cm in the control to 12.00–12.33 cm with 200 ppm ozone in the first season and from 9.60 to 11.73–11.76 cm in the second season with 30 ppm. Cold treatment did not significantly differ relative to the control. Fruit diameter was significantly affected by the interaction, except in seeds soaked in the second season. The evaluated hybrids slightly differed in fruit diameter, with insignificant differences between the application methods of ozone. In the case of total soluble solids vitamin C contents in fruits, the differences were statistically detectable only in the first season. A slight increase due to high ozone concentration was attained, with a favorable effect of seedlings spray compared to seed soaking.

Table 7. Effect of ozone and chilling tolerance pretreatments on morphological and chemical quality characteristics of the fruits of two sweet pepper hybrids during the 2017/18 and 2018/19 seasons.

Treatment		Length (cm)		Diameter (cm)		TSS (%)		Vitamin C (mg 100 g^{-1} FW)	
		Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray	Seed Soaking	Seedling Spray
2017/2018									
Zidenka cv.	Control	9.10 c	8.00 cd	8.33 ab	8.00 b	10.00 a	10.00 ab	650 ab	650 ab
	* Cold	9.60 bc	8.63 c	8.00 b	8.63 ab	10.06 a	10.06 ab	654 a	654 ab
	1 ppm O_3	10.16 ab	8.16 c	9.00 a	8.16 b	9.26 b	9.60 bc	607 b	627 b
	5 ppm O_3	9.00 c	9.03 b	9.00 a	8.06 b	9.10 b	10.06 ab	593 b	654 ab
	10 ppm O_3	9.70 bc	10.13 ab	8.00 a	8.00 b	9.00 bc	10.33 a	585 b	671 a
	20 ppm O_3	13.00 a	11.86 a	9.00 a	9.00 a	10.16 a	10.06 ab	661 a	654 ab
Lirica cv.	Control	8.83 cd	8.46 c	6.50 c	7.00 c	9.10 b	9.10 c	591 b	602 c
	* Cold	8.33 d	9.00 bc	8.00 b	7.00 c	9.50 b	9.50 bc	619 b	621 bc
	1 ppm O_3	10.00 b	10.00 b	8.00 b	9.00 a	10.00 a	10.20 a	650 ab	663 a
	5 ppm O_3	10.00 b	12.00 a	8.10 ab	8.16 b	9.96 ab	9.46 bc	647 ab	615 bc
	10 ppm O_3	10.00 b	11.00 ab	8.83 ab	8.66 ab	10.00 a	10.40 a	650 ab	670 a
	20 ppm O_3	12.33 a	12.00 a	9.00 a	9.33 a	10.26 a	10.40 a	661 a	678 a
Significance		*	*	*	*	*	*	*	*
2018/2019									
Zidenka cv.	Control	10.20 b	10.20 b	7.60 a	7.60 b	8.06 a	8.06 a	645 a	645 a
	* Cold	10.66 ab	9.40 c	8.66 a	7.80 b	7.90 a	8.26 a	632 a	657 a
	10 ppm O_3	9.33 c	10.33 b	8.20 a	8.06 ab	8.06 a	8.83 a	648 a	657 a
	20 ppm O_3	9.66 bc	10.53 b	8.26 a	8.60 a	8.30 a	8.53 a	660 a	658 a
	30 ppm O_3	9.33 c	11.53 a	8.36 a	8.66 a	8.53 a	8.43 a	682 a	679 a
	60 ppm O_3	11.26 a	11.00 ab	8.10 a	7.86 b	8.03 a	9.09 a	680 a	675 a
Lirica cv.	Control	9.60 bc	9.60 c	8.06 a	8.06 ab	8.80 a	8.80 a	656 a	656 a
	* Cold	9.90 b	10.10 bc	8.96 a	8.30 a	8.50 a	8.70 a	665 a	665 a
	10 ppm O_3	10.80 ab	9.76 c	8.73 a	8.66 a	8.50 a	8.43 a	661 a	661 a
	20 ppm O_3	11.13 a	10.20 b	8.76 a	9.00 a	8.70 a	8.80 a	661 a	676 a
	30 ppm O_3	11.73 a	11.76 a	9.13 a	9.26 a	9.23 a	9.30 a	696 a	702 a
	60 ppm O_3	11.66 a	11.30 a	8.20 a	8.46 a	8.83 a	9.53 a	686 a	699 a
Significance		*	*	ns	*	ns	ns	ns	ns

Means followed by a lowercase letter in the same column are not significantly different according to Duncan's multiple range test at 0.05 levels for each season separately. * Cold stress treatment was performed at 4 °C for 36 h. Ozonated water applied as seed soaking or seedling spray compared to the untreated control.

4. Discussion

Cold stress is a serious threat to plant production science. It plays a significant role in crop losses. Harmful effects on plants include poor germination, poor seedling health index, leaf chlorosis, reduced leaf area, wilting, and death of tissue [42]. Pepper plants suffer from cold stress even if they are grown in unheated plastic greenhouses during the cold season [3]. The inside temperature of the plastic greenhouse can reach 5–10 °C during January (Figure 1), owing to the characteristics of polyethylene cover, which allow for rapid loss of stored heat (during daytime) at night [4]. Hence, the morphological, physiological, and biochemical properties of the plants are strongly affected, as reflect by low yield and poor quality. The results obtained in the current study confirm the results reported in [43]. Excessive harm can occur if plants are grown under other stressful conditions, such as salinity, which commonly occurs during winter in arid regions [44]. High soil salinity was shown at the experiment site. Furthermore, the studied hybrids have high yields with good quality when grown in plastic greenhouses and have recently received increased attention from growers. However, their yield and quality are strongly affected by cold stress during January, which can extend from December to February (Figure 7); therefore, they require additional acclimation to cold stress.

Plants exposed to cold stress generally exhibit water stress symptoms [41,45]. Our results indicate that the plants exposed to chilling stress displayed a low percentage of relative water content compared to the control (Table 1), which is considered a good quantitative indicator of water status in the plant. Low RWC values were detected in the control compared to the plants pretreated with cold or ozone before transplanting in the unheated plastic greenhouse. Thus, the pretreatments helped the plant to maintain high RWC valued when the plants were later grown under cold stress. As a result, cell membranes gradually lost their properties and ability to normally transition, which was accompanied by high membrane permeability (Table 4). These results confirm those reported in [46], in which it was found that the grown plants under stress showed low water content, and the cells membranes had high electrolyte leakage.

Plants were reported to suffer from highly stressful conditions when they were exposed to high ozone concentrations [47]. In this case, ozone caused damage in the cellular membrane, altering gene expression and metabolic activities and ultimately causing the death of plant tissues [48]. Wilkinson et al. [49] suggested that such exposure to ozone can cause a decline in carbon transport to the roots and a decrease in nutrient and water uptake, affecting plant stability and leading to a reduction in growth and processes of biomass production. Peng et al. [20] reported the negative impact of O_3 on the productivity of maize crop, with O_3 sensitivity in the following order: photosynthetic physiology > biomass > yield components. The results of the present study indicate that cold acclimation through seed merging or seedling spray by ozonated water at a low dose (30–60 ppm) had several, benefits including a decrease MP (Table 4). Although ozone has been extensively studied as a phytotoxic air pollutant with a harmful effects on the environment and biodiversity [50], little attention has been paid to its beneficial effects on plants. Recently, hormesis, the "dose-dependent response" of different stressors such as temperature and salinity in relation to biological and physiological activities, has been considered [51,52]. However, hormesis of air pollutant generally and ozone specifically in plants requires additional investigations. Hormesis greatly depends on plant species and stressor dose [53]. A low dose of stressor induces defense mechanisms, leading to a positive effect on plant health and performance [52]. Some studies have considered ozone a "eustressor", which, if applied in a suitable concentration and at the appropriate time for a constant period, should improve plant nutritional quality, as reflected by an increase in antioxidant capacity, secondary metabolites, and bioactive compounds, as a tool to cope with the increase in reactive oxygen species (ROS) and oxidative stress as a result to exposure to ozone [30,54]. Marchica et al. [54] reported ozone application in a low dose and for a short period of exposure as an effective method for priming purposes by activation of the phenolic pathway, leading to an increase in the production of phenolic metabolites and increased total antioxidant activity. These outcomes support the concept of ozone as a "eustressor" when applied at a low dose for a short period and reported ozone as a potential tool to improve the quality of sage leaf extracts. These results are supported by those reported by Pellegrini [30], who reported ozone prime as a potential method to increase the beneficial properties of the medicinal herb *Hypericum perforatum* plants at different stages by enhancing upregulation of the reduced/oxidized glutathione ratio (up to about 2-fold higher than the untreated plants), which is related to an increase in peroxidase activity, as well as the accumulation of total phenols, flavonoids and anthocyanins and an increase in antioxidant potential. These potential benefits of ozone in low concentrations on plants were also reported for the development of a rapid and non-destructive method using reflectance spectroscopy for early detection of the responses of crops to ozone [53]. This method was developed to measure the adaptation mechanism of the plant to cope with the ozone effect (in the absence of visible symptoms) by estimating related parameters of photosynthesis, oxidative pressure, and antioxidant capacity. Our results support these findings; most importantly, we used a simple, cost-effective, and ecofriendly method to mitigate cold stress of sweet pepper grown in an unheated plastic greenhouse, which improved fruit productivity and quality. Ozone is considered an ecofriendly and natural method in several fields, including medicine and agriculture, without negative impacts on the environment [55]. The present study indicates that cold acclimation through seed merging or seedling spray by ozonated water at a low dose (30–60 ppm) had several physiological and biochemical benefits. We attempted to explain physiological changes related to ozone priming by estimating the changes in membrane permeability (Table 4). The reduction in electrolyte leakage could protect the cells from damage under environmental stresses, including cold stress. Hence, MP is considered a sensitive stress marker due to the sensitivity of the cellular membrane to various environmental stresses that always cause oxidative damage accompanied by increasing permeability [56]. Plants grown under stress produce ROS through the induction of antioxidant defense systems, which conserve the membranes and other essential substances [57]. Nali et al. [58] suggested that increasing antioxidant levels (both enzymatic and non-enzymatic) taking part in cellular repair processes increases cell wall strength, reducing MP and increasing cell content of water, as reported in our study (Table 4), to reduce stress caused by ROS [59]. A similar response was observed in some leguminous plants [60] and a considerable number of herbaceous species [61]. Plants accumulate additional proline under cold stress (Figure 3) to maintain osmotic adjustment and membrane stability and protect cells from the disruptive effect of free radicals [62,63]. Sufficient indices could explain the favorable effect of low ozone concentration on proline accumulation under cold stress (Figures 2 and 3). Zhang et al. [64] reported that proline accumulates in response to biotic and abiotic stresses. In this case, proline contributes to conserving tissue water, as well as protection of proteins and cellular membranes from osmotic and oxidative stresses [65]. Furthermore, proline helps to maintain osmotic adjustment and membrane stability and decreases the effects of ROS damage as a free radical scavenger [66], improving resistance against chilling [67] and helping to protecting enzymes, biological membranes, and photosynthetic apparatus from oxidative damage [68]. Consequently, the increase in proline content can be considered a good indicator of cold stress resistance.

Moreover, chilling induces oxidative damage in both root and leaf tissues [48]. Ozone can provide acclimation protection against oxidative stress by increasing ascorbate peroxidase (APX) activity (Figures 4 and 5). Yan et al. [69] reported that APX activity depended on ozone dose and the exposure period. In our study, APX activity increased when the seed or seedlings were acclimated to a low ozone dose (30–60 ppm) for a short time, i.e., seed soaking for 1 h or seedling spray by ozonated water. These results are in accordance with the findings of Liu et al. [70], who reported that cold acclimation effectively alleviates oxidative damage and increases photosynthetic capacity. Flowers et al. [71] reported excess bean growth and yield as a result of increasing photosynthesis intensity when the plants were exposed to low ozone concentrations for a short time (15 ppb O3 for 12 h weekly until harvest) in comparison to ozone-free plants. Similarly, an increase in photosynthesis was achieved with low exposure time for 1 h/day (compared to the control), which then decreased with increased exposure time to 4 h/day [72]. The present study supports these findings, confirming that exposure to low ozone concentrations for a short period causes an increase in chlorophyll intensity (Table 3), which is closely connected to photosynthesis [73].

It was noted that pepper plants have a severe problem with pollination and fertilization, as well as a severe reduction in fruits yield and quality under cold stress [3,74]. To solve such a problem, fruit set percentage and seed count inside the fruit were investigated during the coldest time of the season in December and January to determine whether the acclimation effect lasted until the flowering and fruiting stages. Furthermore, we could not find any publications regarding the role of ozone acclimation in the production of sweet pepper under double stresses of cold and salinity in an unheated plastic greenhouse. Our results show that the favorable effect of ozone acclimation exceeded the physiological and biochemical properties during vegetative growth and was prolonged throughout the productivity stage. Accordingly, ozone acclimation significantly augmented flower and fruit numbers, as well as fruit set percentage (Table 5). Additionally, the formed fruits had high seeds count and consequently produced larger fruits with higher quality, in addition to a significant increase in the marketable and total yields compared to the control (Table 6 and Figure 7).

5. Conclusions

The present work provides evidence that ozone plays a beneficial role in plant resistance to cold, which be achieved when ozone is applied in a low concentration for a brief period during early plant development as seed soaking or seedling foliar spray. Ozonated water with concentrations ranging from 0 to 60 ppm was applied to two hybrids of sweet pepper. The effectiveness of treatments was evaluated during plant production under unheated greenhouse conditions in the winter season. The results indicate that ozone treatment at 30 ppm as seed soaking or seedling spray is a promising, simple, practical, and ecofriendly strategy without harmful effects on the environment.

Ozone significantly increased plant leaf area, the contents of proline, and ascorbate peroxides activity, which promote the mechanism of plant defense against cold stress, as reflected by increased contents of chlorophyll (SPAD) and relative water content but reduced and preferable membrane permeability. Furthermore, vegetative growth and fruit set were increased, subsequently improving, early, total, and marketable pepper fruit yields, as well as fruit quality under cold stress. Finally, the appropriate application of ozone with the correct right dose, application time/period, and plant type could contribute to enhanced plant resistance to stresses and lead to improved adaption to climate changes in different regions. Additional detailed studies are needed to examine the role of ozone application to different plant species under various stress conditions.

Author Contributions: Conceptualization, M.A.S.-E., S.E., M.S. and Z.M.Y.; Data curation, M.A.S.-E., A.M.Y., M.S. and Z.M.Y.; Formal analysis, M.A.S.-E., A.A.E., A.M.Y., S.E. and Z.M.Y.; Investigation, A.M.Y., M.S. and Z.M.Y.; Methodology, A.M.Y.; Project administration, Z.M.Y.; Resources, M.A.S.-E.; Software, A.A.E.; Supervision, S.E., M.S. and Z.M.Y.; Validation, A.A.E., S.E., M.S. and Z.M.Y.; Visualization, A.M.Y., S.E. and Z.M.Y.; Writing—original draft, M.A.S.-E., A.A.E., A.M.Y., S.E., M.S. and Z.M.Y.; Writing—review and editing, A.A.E. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported predominantly by WATERAGRI (European Union Horizon 2020 research and innovation programme under Grant Agreement Number 858735).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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