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Corn Steep Liquor Application Improves Pepper (*Capsicum annum* L.) Tolerance to Salinity

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Abstract: Salinity, caused by irrigation with water containing high salt concentrations, excessive fertilization, or the loss of leaching capacity in some soils, is a serious problem on a global scale. Its incidence leads to osmotic and specific effects, as well as an imbalance in nutrient uptake that hinders the growth of most crops. Biostimulants can improve salt tolerance by reducing the uptake and accumulation of toxic ions. Corn steep liquor (CSL) is a byproduct of corn cleaning and maceration. This study investigates whether CSL application induces adaptive responses in pepper (*Capsicum annuum* L.) plants cultivated under saline conditions. Four treatments were carried out with pepper plants in a culture chamber: irrigation with Hoagland nutrient solution; irrigation with 100 mM NaCl in the Hoagland nutrient solution; irrigation with 100 mM NaCl in the Hoagland nutrient solution and the foliar application of CSL at 5 mL L^{-1} every 7 days; and irrigation with 100 mM NaCl in the Hoagland nutrient solution and root application of CSL at 5 mL L^{-1} every 7 days. The beneficial effect of CSL in reducing the phytotoxicity of salt stress was found to be due to an improvement in the photosynthetic efficiency and a reduction in the generation of reactive oxygen species. Thus, the increase in MDA concentration due to saline treatment is less when applying CSL, which is 3.5 times less when it is performed via the foliar route and 4.6 times if the treatment is on the root. The results show that CSL application increased the aerial biomass and leaf area under saline conditions through physiological mechanisms that varied depending on the application method.

Keywords: antioxidant activity; biostimulant; ionic toxicity; oxidative stress; proline

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1. Introduction

Salinity is a serious problem in global agriculture, affecting nearly 1 billion hectares of land, which is approximately 20%, almost half of the irrigated arable land worldwide [1]. The low quantity and quality of water available for irrigation makes it necessary to use groundwater and unconventional sources with high concentration of salts. As such, a progressive salinization of the soil develops, especially in arid areas with irrigated crops [2]. The excessive use of fertilizers and the reduced leaching capacity of certain soils have also contributed to this problem [3]. The salinity of irrigation water and soil hinders the growth of most crops due to the inhibition of water uptake through the osmotic effects caused by the increased salt concentration in the root zone. It also leads to an excessive uptake of Na^+ and Cl^-, resulting in specific ionic toxicity [4]. Furthermore, the high concentrations of these ions, Na^+ and Cl^-, in the root zone, disrupt the uptake of cations such as K^+ and

Ca^{2+}, as well as anions such as NO^{3-} and $PO_4{}^{3-}$ [5]. The high concentrations of Na^+ and Cl^- also affect other processes, including water relations, light capture, CO_2 assimilation and antioxidant capacity, among others, ultimately resulting in reduced growth, biomass, and crop yield [6–11].

The use of biostimulants could prove to be an effective tool in reducing the toxic effect of salinity in plants, partly due to a reduction in the uptake and accumulation of Na^+ and Cl^- ions [12]. Thus, the application of chitosan-based salicylic acid nanocomposite in a vineyard (*Vitis vinifera* cv 'Sultana') [13], the foliar application of 24-epibrassinolide to pepper (*Capsicum annuum* L.) [14], the treatment of basil (*Ocimun basilicum* L.) with a hydrolyzed animal protein-based biostimulant [15], the application of a graminaceae-derived protein hydrolysate and its fractions to lettuce (*Lactuca sativa* L.) [16], the addition of a protein hydrolysate of plant origin to spinach (*Spinacia oleracea* L.) [17], the application of the hydroalcoholic extracts of brown algae (*Sargassum* spp.) to tomato (*Solanum lycopersicum* L.) [18], and the addition of *Ulva intestinalis* (L.) extract to bean (*Phaseolus vulgaris* L.) [19] have been found to improve production and quality under saline conditions. Corn steep liquor (CSL) is a byproduct obtained from the cleaning and maceration of corn during wet milling. Although its use requires appropriate treatment to avoid environmental issues [20], it holds great promise in terms of the circular economy and sustainability [21]. It contains high amounts of proteins, amino acids, minerals, vitamins, reducing sugars, organic acids, enzymes, and other substances that promote plant growth. Its application to soil improves the utilization of macronutrients by promoting the growth of bacteria that contribute to nitrogen (N) fixation and phosphorus (P) solubilization [22]. In lettuce hydroponics, the use of CSL favored microbial development that protected the root system [23,24]. In soybean (*Glycine max*) crops, treatment with 1% CSL favored germination, growth and precocity due to an increase in the uptake and transport of nutrients [25].

To meet the expectations generated by the biostimulant sector, it is necessary to establish the composition and mechanism of action of each product. In a previous study on the role of two CSL products, with different stabilization methods, in pepper (*Capsicum annuum* L.) plants, a better response was found with the so-called CSL-B, which undergoes filtration to remove suspended solids and stabilization [26]. Its composition (%, g g^{-1} fresh product) includes free amino acids (5.0–6.0), total organic matter (40), total humic extract (30), fulvic acids (30), total N (3.0), ammoniacal N (0.3), organic N (2.7), potassium, K_2O, (2.5) and P, P_2O_5, (3.0). Its mode of action is related to regulation, hormone synthesis, and the stimulation of C and N metabolism [26]. Despite being a widely studied product with many beneficial applications, it is unknown whether the application of CSL induces adaptive responses in pepper plants grown under challenging conditions such as salt stress. This study examines the mechanisms of action of this type of CSL in this crop under saline conditions and the effect of the application method, either root or foliar.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Pepper plants (*Capsicum annuum* cv. Alycum) that were 45 days old were planted in 2 L pots filled with a substrate comprising perlite and peat at a ratio of 1:3. The plants were placed in a growth chamber with a temperature of 29 °C and a relative humidity (RH) of 60% during the day (16 h), with a photosynthetic photon flux density (PPFD) of 450 µmol photons m^{-2} s^{-1}, and 20 °C and 80% RH during the night. Fertigation was carried out using a Hoagland nutrient solution composed of 4 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mM NaH_2PO_4, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$, 0.1 µM Na_2MoO_4, 125 µM Fe-EDDHA, and 50 µM H_3BO_3 (pH 5.8). A weekly irrigation was performed with a volume ranging from 50 to 100 mL, according to the size of the plants.

2.2. Experimental Design

Seven days after transplantation, the following treatments were applied: T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl

in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1}; and T4: 100 mM NaCl in the Hoagland nutrient solution with a root application of CSL at 5 mL L^{-1}. The CSL product, both via foliar and root application, was applied four times at 7-day intervals from the beginning of the treatments. In the growth chamber, two blocks were prepared with four treatments in each one and eight plants per treatment with random distribution. Seven days after the last CSL application, plant growth was determined, physiological status was evaluated, indicators of oxidative stress and antioxidant components were analyzed, and the specific toxicity was established.

2.3. Aerial Biomass and Leaf Area

The measurement of the leaf area was carried out using a LI-COR optical reader, model LI-3000A (IRGA: LICOR Inc., Lincoln, NE, USA). For the determination of dry matter (DM), the leaves were dried by radiation and forced convection in an oven. Leaf samples for determining oxidative indicators and antioxidant compounds were stored at −40 °C.

2.4. Gas Exchange Measurements

The measurements were performed using the LICOR 6800 Portable Photosynthesis System (IRGA: LICOR Inc., Lincoln, NE, USA), an infrared gas analyzer. A fully developed leaf, in an intermediate situation, was selected from every eight plants per treatment. The calibration conditions were 500 mol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), a CO_2 concentration of 400 mol mol^{-1}, a leaf temperature of 30 °C, and 60% RH. Net photosynthetic rate (A), transpiration rate (E), and stomatal conductance (r) were simultaneously recorded. The data were analyzed using the Photosyn Assistant software (IRGA: LICOR Inc., NE, USA). To estimate the instantaneous water use efficiency (WUE), the relationship between A and E was used [27].

2.5. Fluorescence of Chlorophyll a (Chl a)

The fluorescence kinetics of Chl a were determined, using a Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK), for completely developed leaves from the center of the plant. Red light (650 nm) with an intensity of 3000 µmol photons m^{-2} s^{-1} was used to induce the fluorescence phases, and the JIP test was employed for analysis [27]. The parameters determined were the maximum quantum yield of primary photochemistry (Fv/Fm), performance index (PI_{ABS}), proportion of active reaction centers (RC/ABS), and electron output (1-Vj) [27].

2.6. Oxidative Stress

The extraction of malondialdehyde (MDA), $C_3H_4O_2$, was performed on homogenized fresh plant material in a mortar with 5 mL of 50 mM buffer (0.07% $NaH_2PO_4 \cdot 2H_2O$ and 1.6% $Na_2HPO_4 \cdot 12H_2O$). The mixture was then centrifuged at 20,000× *g* (Heraeus Sepatech Biofuge 17RS, Hanau, Germany) for 25 min. Next, 1 mL of the supernatant was mixed with 4 mL of 20% trichloroacetic acid (CCl_3COOH) containing 0.5% thiobarbituric acid ($C_4H_2N_2O_2S$). The resulting mixture was heated at 95 °C for 30 min and then rapidly reduced. This sample was then centrifuged at 10,000× *g* for 10 min (Heraeus Sepatech Biofuge 17RS, Hanau, Germany). The absorbance of the supernatant was measured at 532 nm. The non-specific absorption at 600 nm was subtracted from the obtained reading [28].

The concentration of hydrogen peroxide (H_2O_2) was measured by colorimetry according to [29]. For this, fresh plant material was homogenized in cold acetone (C_3H_6O). A 1 mL aliquot of the extract was mixed with 200 µL of 0.1% titanium dioxide (TiO_2) in 20% H_2SO_4 (*v*:*v*) and the mixture was centrifuged at 6000× *g* for 15 min (Heraeus Sepatech Biofuge 17RS, Hanau, Germany). The intensity of the yellow color of the supernatant was measured at 415 nm, and the concentration of H_2O_2 was calculated from its corresponding standard curve.

The superoxide (O_2^-) concentration was determined by colorimetry [30], and was calculated from a standard curve.

2.7. Antioxidant Activity

2.7.1. Antioxidant Compounds (Total Phenols, Ascorbate, Glutathione)

Total phenols were extracted from plant tissue with methanol, CH_4O. The content was determined based on the absorbance at 765 nm using the Folin–Ciocalteu reagent [31]. The phenol concentration was derived from a standard curve of caffeic acid, $C_9H_8O_4$.

For the extraction and quantification of ascorbate (AsA), the method described by [32] was used, which is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acidic solution. Frozen plant material (0.5 g) was homogenized in 5 mL of 5% (*w*/*v*) metaphosphoric acid, HPO_3. The mixture was then centrifuged at 16,000× *g* at 4 °C for 15 min (Heraeus Sepatech Biofuge 17RS, Hanau, Germany). Then, 0.2 mL of the supernatant was added to a test tube along with 0.5 mL of 150 mM sodium phosphate buffer (pH 7.5) and 0.1 mL of distilled water. The mixture was shaken and incubated at room temperature in darkness for 10 min. Subsequently, 0.1 mL of 0.5% (*w*/*v*) N-ethylmaleimide, $C_6H_7NO_2$, 0.4 mL of 44% (*v*/*v*) orthophosphoric acid, H_3PO_4, 0.4 mL of 4% (*w*/*v*) 2,2′-bipyridine, $C_{10}H_8N_2$, in 70% ethanol, C_2H_6O, and 0.2 mL of 3% (*w*/*v*) $FeCl_3$ were added to the tube. After shaking, the mixture was incubated at 40 °C in darkness for 40 min. Finally, the absorbance was measured at 525 nm against an ascorbate standard curve.

The concentration of reduced glutathione (GSH), $C_{10}H_7N_3O_6S$, was determined as detailed by [32], based on the specificity of glutathione reductase (GR) for oxidized glutathione (GSSG). First, extraction was carried out using 0.5 g of fresh material homogenized in 5 mL of 5% (*v*/*v*) metaphosphoric acid, HPO_3. The sample was filtered and centrifuged at 16,000× *g* for 15 min at 0 °C (Heraeus Sepatech Biofuge 17RS, Hanau, Germany). For the quantification of total GSH, a reaction mixture was prepared with 50 µL of the extract and 250 µL of 50 mM Heppes-HCl buffer, pH 7.6, containing 330 mM betaine, $C_5H_{11}NO_2$, and 150 µL of 10% (*v*/*v*) sulfosalicylic acid, $C_7H_6O_6S$. Subsequently, in a test tube, 150 µL of the reaction mixture, 700 µL of 0.3 mM NADPH, and 100 µL of 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The mixture was shaken, and after a 4 min incubation, 50 µL of GR (10 U/mL) were added. Finally, the samples were read at 412 nm against a GSH standard curve.

2.7.2. Antioxidant Tests FRAP and TEAC

The ferric ion reducing antioxidant potential (FRAP) assay was performed using the FRAP reagent, composed of 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 20 mM $FeCl_3$ in 0.25 M sodium acetate, CH_3COONa, pH 3.6. One hundred microliters of the extract obtained by homogenizing leaf material with 10 mL of methanol, CH_4O, was added to 2 mL of the FRAP reagent. This mixture was maintained at 20 °C for 5 min. The absorbance was measured at 593 nm against a standard curve of 25–1600 µM Fe^{3+} obtained from a 25 mM solution of ferrous sulfate, $FeSO_4$ [33].

The Trolox equivalent antioxidant activity (TEAC) test was conducted using a modified version of the procedure described by [34]. First, 7 mM 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was mixed with 2.45 mM potassium persulfate, $K_2S_2O_8$, to generate the $ABTS^+$ cation. The resulting mixture was incubated in the dark at room temperature for 16 h. Then, the $ABTS^+$ solution was diluted with methanol, CH4O, and calibrated at a wavelength of 734 nm. A 100 µL aliquot of leaf extract (0.5 g in 10 mL of CH_4O) was vigorously mixed with 3.9 mL of diluted $ABTS^+$ solution and then kept in the dark at room temperature for 6 min, followed by the immediate measurement of the absorbance at 734 nm. The samples were compared to a standard curve of 0–15 µM Trolox subjected to the same procedure.

2.8. Proline

The concentration of free proline, $C_5H_9NO_2$, in the leaves was determined using the method of [35].

2.9. Na^+, Cl^-, and K^+ Concentrations

Leaf samples were mineralized according to the method described by [36]. Digestion of dried leaves (0.2 g) was carried out in 30% HNO_3 and H_2O_2 at 300 °C. The determination of the Na^+, Cl^-, and K^+ concentrations was performed using ICP-OES (Perkin-Elmer Optima 8300, Waltham, MA, USA).

2.10. Statistical Analysis

All analyses were carried out in triplicate, and the results were statistically evaluated by analysis of variance (ANOVA) with a 95% confidence interval. There were no differences between blocks. Differences between the treatment means were compared using Fisher's least significant difference (LSD) test at a probability level of 95%. The levels of significance were expressed as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS—not significant.

3. Results

3.1. Aerial Biomass and Leaf Area in Pepper Plants (Capsicum annuum, cv. Alycum)

The salinity treatment caused a reduction in aerial biomass and leaf area. Plants treated with 100 mM NaCl exhibited a lower aerial biomass (35% of fresh weight and 48% of dry weight) and leaf area (43%) compared to the control plants. The application of the corn steep liquor (CSL) product to plants treated with 100 mM NaCl, either via foliar or root application, was beneficial in minimizing the decrease in aerial biomass and leaf area due to salinity. Thus, the values of aerial biomass, fresh or dry, and leaf area in these CSL treatments, ranged from 50% to 70% of the values of the control plants. Although the results did not differ significantly among the CSL treatments, the root application of CSL led to higher values of biomass and leaf area than the foliar treatment did (Table 1).

Table 1. Aerial biomass production and leaf area in pepper plants (*Capsicum annuum*, cv. Alycum), 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	Aerial Fresh Biomass (g $plant^{-1}$)	Aerial Dry Biomass (g $plant^{-1}$)	Leaf Area (cm^2)
T1	55 ± 7 a	5.2 ± 0.7 a	1200 ± 200 a
T2	19 ± 2 c	2.5 ± 0.2 c	510 ± 50 c
T3	27 ± 2 b	3.1 ± 0.3 b	780 ± 60 b
T4	30 ± 2 b	3.2 ± 0.2 b	830 ± 40 b
p-value	***	***	***

The values indicate the means ± standard deviations (SDs) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments at $p < 0.05$. In the ANOVA, the significance level is shown by *** ($p < 0.001$).

3.2. Gas Exchange Parameters in Pepper Plants (Capsicum annuum, cv. Alycum)

The values of the rate of photosynthesis (A), the rate of transpiration (E), and the water use efficiency (WUE) were significantly reduced in plants exposed to 100 mM NaCl under the experimental conditions used. The greatest reduction occurred in the case of A, its value under saline conditions being 20% of that of the control plants. The CSL treatments, particularly the root application, mitigated this effect, with the value of A being 56% of that of the control plants. Plants subjected to salt stress experienced a significant increase in stomatal resistance (r; value equal to 141% of that of the control plants) due to a generalized stomatal closure, to prevent excessive water loss. The CSL treatments limited the magnitude of this increase, especially when applied to the roots, with the value being equal to 127% of the control plants (Table 2). Stomatal closure is thought to be a fast mechanism of adaptation to water stress and is vital for water conservation in plants. However, in this study, as time passed, a considerable reduction in the net rate of

photosynthesis (Table 2) resulted in a significant decrease in biomass production in these stressed plants (Table 1).

Figure 1 displays plants corresponding to the different treatments. The growth reduction caused by the salinity treatment can be observed, as well as the recovery effect due to the application of CSL, particularly through root application.

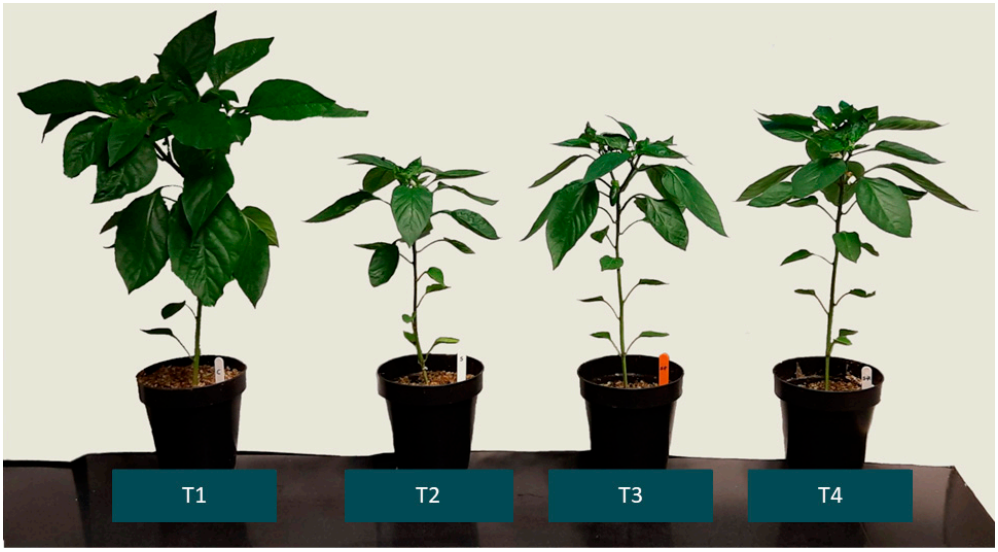

Figure 1. Pepper plants (*Capsicum annuum*, cv. Alycum) subjected to different treatments at the end of the experiment. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Table 2. Parameters of photosynthetic efficiency in pepper plants (*Capsicum annuum*, cv. Alycum), 7 days after the treatments commenced. A: net assimilation of CO_2, E: leaf transpiration; r: stomatal resistance; WUE: water use efficiency. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	A (µmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	r (s cm^{-1})	WUE
T1	6.1 ± 0.7 a	0.67 ± 0.05 a	6.6 ± 0.4 c	9.0 ± 0.5 a
T2	1.3 ± 0.3 d	0.34 ± 0.04 c	9.3 ± 0.3 a	4.0 ± 0.6 d
T3	2.2 ± 0.2 c	0.40 ± 0.04 bc	8.8 ± 0.4 ab	5.3 ± 0.2 c
T4	3.4 ± 0.2 b	0.44 ± 0.06 b	8.4 ± 0.5 b	7.7 ± 1.4 b
p-value	***	***	***	***

The values indicate the means ± standard deviations (SDs) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments ($p < 0.05$). In the ANOVA, the significance level is indicated by *** ($p < 0.001$).

3.3. Chl a Fluorescence in Pepper Plants (Capsicum annuum, cv. Alycum)

The control plants had an Fv/Fm value of 0.849. The salinity treatment caused a reduction in the Fv/Fm value to 93% of the value of the control plants (Table 3). The application of the CSL product mitigated this reduction and the values were equal to 98% of that of the control plants. The rest of the indices determined showed a similar behavior, except for the 1-Vj value. In this case, no significant differences were found among the treatments.

Table 3. Chl a fluorescence in pepper plants (*Capsicum annuum*, cv. Alycum) 7 days after the treatments commenced. Fv/Fm, quantum yield of primary photosynthesis; RC/ABS, active reaction centers of photosystems; PI_{ABS}, photosynthetic performance index; 1-Vj, electron output from photosystem II. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	Fv/Fm	RC/ABS	PI_{ABS}	1-Vj
T1	0.849 ± 0.010 a	0.94 ± 0.10 a	9.9 ± 1.1 a	0.69 ± 0.01 a
T2	0.793 ± 0.013 c	0.71 ± 0.10 b	7.2 ± 1.2 b	0.70 ± 0.01 a
T3	0.830 ± 0.006 b	0.86 ± 0.04 a	9.2 ± 0.5 a	0.70 ± 0.01 a
T4	0.829 ± 0.006 b	0.87 ± 0.04 a	9.9 ± 1.2 a	0.72 ± 0.02 a
p-value	***	*	*	NS

The values indicate the means ± standard deviations (SDs) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments ($p < 0.05$). In the ANOVA, the significance level is indicated by * ($p < 0.05$); *** ($p < 0.001$); NS—no significant.

3.4. Oxidative Stress

The values of the oxidative stress indicators analyzed, MDA, O_2^-, and H_2O_2, were significantly greater for the plants exposed to 100 mM NaCl than for the control plants under the experimental conditions employed, especially the values of O_2^- (equal to 350% of that the control plants) and MDA (180%). The application of CSL reduced the values of these parameters, especially those of MDA (124% of the control plants in the foliar treatment and 118% in the root treatment) (Table 4).

Table 4. Oxidative stress indicators in peppers plants (*Capsicum annuum*, cv. Alycum) 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	MDA (μM g^{-1} FW)	O_2 (μg g^{-1} FW)	H_2O_2 (μg g^{-1} FW)
T1	3.4 ± 0.2 c	4 ± 1 c	32 ± 3 c
T2	6.2 ± 0.4 a	14 ± 1 a	45 ± 4 a
T3	4.2 ± 0.1 b	8 ± 1 b	39 ± 4 b
T4	4.0 ± 0.2 b	8 ± 1 b	39 ± 3 b
p-value	***	***	***

The values indicate the means ± standard deviations (SDs) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments ($p < 0.05$). In the ANOVA, the significance level is indicated by *** ($p < 0.001$).

3.5. Antioxidant Compounds in Pepper Plants (Capsicum annuum, cv. Alycum)

The salinity treatment increased the concentrations of the antioxidant compounds, especially phenols (180% of the value of the control plants) and ascorbate (175%) (Table 5). Under saline conditions, the foliar application of CSL increased the levels of these compounds, while the root application decreased them.

Table 5. Antioxidant compounds in pepper plants (*Capsicum annuum*, cv. Alycum) 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	Phenols (mg g^{-1} FW)	Ascorbate (mg g^{-1} FW)	Glutathione (mg g^{-1} FW)
T1	2.1 ± 0.1 c	0.04 ± 0.01 c	0.08 ± 0.01 c
T2	5.9 ± 1.1 b	0.11 ± 0.01 b	0.10 ± 0.01 b
T3	7.0 ± 1.1 a	0.14 ± 0.01 a	0.14 ± 0.02 a
T4	4.8 ± 1.1 b	0.09 ± 0.01 b	0.10 ± 0.01 b
p-value	***	***	***

The values indicate the means ± standard deviations (SDs) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments ($p < 0.05$). In the ANOVA, the significance level is indicated by *** ($p < 0.001$).

The concentrations of these antioxidant compounds show a trend similar to that of the antioxidant capacity determined by the ferric ion reducing antioxidant potential (FRAP) and Trolox equivalent antioxidant activity (TEAC) tests, which indicate the overall antioxidant activity of the plants. The values yielded by these tests were the highest for the plants subjected to salinity and those receiving the foliar application of CSL under saline conditions, especially for the FRAP test. The values of the TEAC antioxidant test for the plants exposed to CSL applied via the roots are lower than those of the plants grown under saline conditions without CSL treatment (Figure 2).

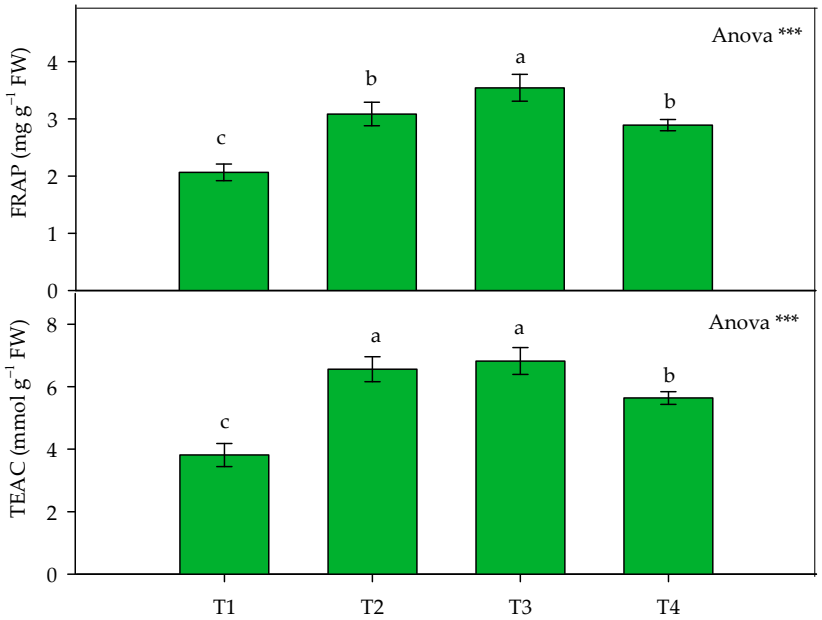

Figure 2. Values of the ferric ion reducing antioxidant potential (FRAP) and Trolox equivalent antioxidant activity (TEAC) tests for pepper plants (*Capsicum annuum*, cv. Alycum), 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days. In the ANOVA, the significance level is represented by $p < 0.001$ (***). The different lowercase letters indicate significant differences ($p < 0.05$) between the means, as established by Fisher's least test (LSD). The values indicate the means ± standard deviations (SDs) (n = 8).

3.6. Proline Concentration in Pepper Plants (Capsicum annuum, cv. Alycum)

Proline levels were significantly higher under saline conditions, underlining that proline is an indicator of this type of stress (Figure 3). Control plants and those treated with CSL under saline conditions, especially via the root, showed the lowest proline concentrations.

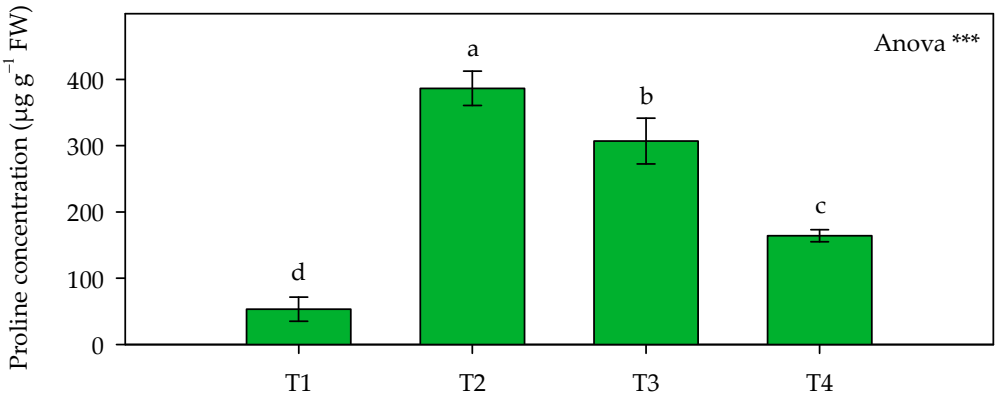

Figure 3. Foliar proline concentrations in pepper plants (*Capsicum annuum*, cv. Alycum), 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with the root application of CSL at 5 mL L^{-1} every 7 days. In the ANOVA, the significance level is represented by $p < 0.001$ (***). The different lowercase letters indicate significant differences ($p < 0.05$) between the means, as established by Fisher´s least test (LSD). The values indicate the means ± standard deviations (SDs) (n = 8).

3.7. Specific Toxicity in Pepper Plants (Capsicum annuum, cv. Alycum)

The salinity treatment raised the leaf concentrations of Na^+ and Cl^-, and decreased that of K^+. The foliar application of CSL under saline conditions diminished the leaf concentrations of Na^+ and Cl^-. The root applications of CSL raised the leaf concentration of K^+ (Table 6).

Table 6. Foliar concentrations of the Na^+, Cl^-, and K^+ ions in peppers plants (*Capsicum annuum*, cv. Alycum), 7 days after the treatments commenced. T1: Hoagland nutrient solution; T2: 100 mM NaCl in the Hoagland nutrient solution; T3: 100 mM NaCl in the Hoagland nutrient solution with the foliar application of CSL at 5 mL L^{-1} every 7 days; and T4: 100 mM NaCl in the Hoagland nutrient solution with root application of CSL at 5 mL L^{-1} every 7 days.

Treatments	Na^+ (mg g^{-1} DW)	Cl^- (mg g^{-1} DW)	K^+ (mg g^{-1} DW)
T1	2.4 ± 0.5 c	2.3 ± 0.5 c	64.3 ± 2.4 a
T2	35.9 ± 3.2 a	36.1 ± 4.6 a	44.8 ± 1.2 c
T3	15.6 ± 1.9 b	16.9 ± 2.0 b	44.8 ± 0.6 c
T4	34.7 ± 4.6 a	37.0 ± 3.3 a	55.3 ± 3.1 b
p-value	***	***	***

The values indicate the means ± standard deviations (SD) (n = 8). The differences between treatments were analyzed with Fisher's least significant difference test (LSD; $p = 0.05$); distinct letters in the same column show significant differences between treatments ($p < 0.05$). In the ANOVA, the significance level is indicated by *** ($p < 0.001$).

4. Discussion

The corn steep liquor (CSL) treatment, especially via root application, significantly improved the growth of pepper (*Capsicum annuum* L.) plants treated with 100 mM NaCl under the prevailing experimental conditions. The application of CSL to the roots of bean (*Phaseolus vulgaris* L.) plants was found to enhance plant growth [25]. The application of biostimulants with different proportions of humic acids and fulvic acids in tomato (*Solanum*

lycopersicum L.) under saline stress conditions improves growth, increasing fresh and dry matter [37]. The application of a biostimulant amino acid to a salt-resistant variety of basil (*Ocimun basilicum* L.) improved production under saline conditions [15]. Treatment with a hydrolyzed protein of plant origin enhanced the lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea* L.) production under moderate salinity, although its effect varied depending on its molecular fraction [16,17].

Under environmental stress, the significant inhibition of photosynthesis is commonly observed [6,7,9–11]. However, in some plant species, the application of biostimulants can reverse this inhibition and restore normal plant growth [12,13,38,39]. In certain cases, these treatments with biostimulants can prevent the complete closure of stomata under stress conditions, thereby promoting the maintenance of photosynthetic activity in plants [38]. The results obtained here verify that the CSL product, particularly via root application, would act in this manner, as its use increased A, E, and WUE, while reducing stomatal resistance (r) compared to plants under salinity stress (Table 2). This effect can also be observed, although to a lesser extent and with less significance, in stressed plants receiving the foliar application of CSL (Table 2). Similar findings were reported for tomato seedlings treated with a hydroalcoholic extract of *Sargassum* spp. under saline conditions [18].

When there is metabolic disturbance, the plants produce fluorescence to dissipate excess energy and prevent damage [27]. The photosynthetic efficiency can be deduced from the value of the quantum yield of primary photosynthesis (Fv/Fm). In healthy plants or those not subjected to intense stress, the Fv/Fm value is typically around 0.85 [27]. In the experimental conditions used here, the control plants had an Fv/Fm value of 0.849. The salinity treatment caused a reduction in the Fv/Fm values, indicating increased chlorophyll a fluorescence and thus high salt stress [27]. The analysis of chlorophyll a fluorescence provides several indices that define plant vitality. The RC/ABS ratio is an essential parameter to evaluate the electron transport chain in photosystems. High values of this indicator correspond to a higher proportion of active reaction centers [27]. The PI_{ABS} index represents the photosynthetic performance functionality of the photosystems. The 1-Vj value denotes electron leakage, particularly from photosystem II [27]. Plants treated with CSL under saline conditions show a mitigated decrease in Fv/Fm, with values above 0.8, suggesting improved adaptation (Table 3). This is due to the enhanced protection and activation of the photochemical process under stress conditions. Intracellular CO_2 availability would have also increased due to reduced stomatal closure, leading to the greater availability of the endogenous electron acceptor, NADP, and thereby reducing the electron transfer to oxygen and, consequently, ROS formation. Additionally, the net photosynthesis rate increased, which contributed to increased biomass production under these stress conditions (Table 1). The rest of the photochemical activity and vitality indices of the plants suggest that, under saline stress conditions, the application of the CSL product, both foliar and via the roots, improved the coupling of the different components of the photochemical stage, hence improving the efficiency of the conversion of light energy into chemical energy, therefore increasing plant vitality. Thus, the RC/ABS and PI_{ABS} indices had their highest values in the control plants and those treated with CSL under saline conditions (Table 3). These results also indicate that the electron loss in the photochemical phase of photosynthesis was reduced by CSL application to plants suffering salt stress, decreasing the formation of reactive oxygen species (ROS) [27].

Saline stress also results in the accumulation of ROS, such as H_2O_2 and O_2^-, which drastically disrupt metabolic homeostasis and affect the integrity of the cell membrane [6,8,10,18]. The reduction in ROS accumulation is crucial for plant survival under saline stress conditions; thus, oxidative metabolism has long been used as an indicator of the resulting damage [6–11]. Plants possess ROS detoxification mechanisms to prevent damage, which can be categorized into enzymatic systems and non-enzymatic systems consisting of antioxidant compounds (phenols, glutathione, flavonoids, ascorbic acid, anthocyanins, etc.) The increase in antioxidant enzymes and compounds under abiotic stress, such as water and salinity stress, depends on the species, cultivar, plant development stage, and metabolic state, as well as

on the intensity and length of the stress [7,9,11]. In the face of the abiotic stresses, biostimulants have been shown to reduce cellular oxidative damage, including the peroxidation of cell membrane lipids, in many plant species. This is achieved through the regulation of antioxidant defenses and the decrease in ROS levels in plants [12,38,39]. In this regard, the concentration of MDA serves as an indicator of lipid membrane peroxidation, and an increase in its values suggests the excessive presence of ROS [7,9,11]. The control plants exhibited the highest values of aerial biomass and leaf area, as well as the lowest levels of leaf MDA (Tables 1 and 4). The plants treated with CSL under saline conditions had slightly higher MDA values, while the highest values were found in the plants experiencing saline stress without CSL application. These findings are in agreement with the foliar concentrations of H_2O_2 and O_2^- (Table 4). When different proportions of humic acids and fulvic acids were applied to tomato plants suffering saline stress, it was found that a reduction in the MDA content improved the response to salinity [37].

One of the mechanisms by which biostimulants improve resistance to abiotic stresses is the induction of the plant's antioxidant defenses, both enzymatic and non-enzymatic [12,38,39]. In this study, the salt treatment induced a significant rise in the abundance of the analyzed antioxidant compounds (phenols, ascorbate, glutathione), especially for plants that received the CSL product via foliar application (Table 5). These results can be interpreted as an attempt to mitigate the oxidative damage caused by adverse growth conditions in pepper plants [7,9,11].

Compounds such as proline are often good indicators of resistance to saline stress, as they frequently play a role as osmoprotectants, osmoregulators, and antioxidants that protect against the generation of ROS [7]. The degradation of proline by the enzyme proline dehydrogenase involves oxygen (O_2) consumption, which reduces the likelihood of ROS generation. This may have happened when CSL was applied to salt-stressed plants, as the levels of ROS were lower than when CSL was not applied (Table 4). Therefore, CSL could act at the root level, enhancing ion selectivity by regulating the uptake of Na^+ and Cl^- and/or their accumulation in root tissues. As such, the translocation of these ions to the aerial part would be reduced, leading to an increased accumulation of K^+ (Table 6). These results suggest that, under the conditions of this experiment, proline was an indicator of plant stress, rather than inducing resistance to saline stress. In a study conducted with tomato and commercial formulations of biostimulants containing different proportions of humic and fulvic acids, an increase in the proline content was observed under salinity [37]. Similar results were obtained for wheat (*Triticum aestivum* L.) seedlings treated with the extracts of the brown alga *Macrocystis pyrifera* [40] and in tomato seedlings treated with a hydroalcoholic extract of Sargassum spp. under saline conditions [18].

According to some authors, the additional supply of organic constituents (e.g., amino acids) and/or hormones (e.g., cytokinins) may enhance ionic selectivity in roots, leading to a reduction in the ionic toxicity of NaCl in the aerial part of plants [12]. The reduction in the foliar concentrations of Na^+ and Cl^- ions (due to CSL application to leaves) and the increase in the foliar concentration of K^+ (when applied to roots), along with the activation of other resistance processes, would explain the enhanced growth of plants receiving CSL under saline stress. The formulations of microalgae and cyanobacteria extracts promoted salinity tolerance in tomato by enhancing the enzymatic antioxidant activity, root growth, and nutrient uptake [41]. The application of a plant-based protein to lettuce reduced the ionic toxicity under moderate salinity when its molecular fraction was intermediate (between 1 and 10 kDa) [16].

5. Conclusions

Under saline stress conditions, the application of corn steep liquor (CSL) resulted in a significant increase in biomass production in the aerial part, and in the leaf area. Therefore, the reduction in salinity stress caused by CSL can be mainly attributed to physiological mechanisms that varied depending on the application method. In the case of the foliar application of CSL, there was an induction of the antioxidant capacity and the synthesis

of antioxidant compounds. This, together with the reduction in ROS formation due to the increased photochemical and photosynthetic efficiency and a decrease in the foliar concentrations of Na^+ and Cl^-, would have prevented oxidative damage and growth reduction. The root application of CSL maintained the photochemical activity and stimulated the photosynthetic efficiency. Along with a higher foliar concentration of K^+ and reduced stomatal closure under saline stress, this allowed a high rate of net photosynthesis and reduced ROS generation, as well as counteracting the phytotoxic effect of Na^+ ions. Thus, the root application of CSL could contribute to improving pepper production under salinity stress by reducing the K^+ fertilization. However, it is necessary to evaluate the effect of this treatment on the production and quality of pepper (*Capsicum annum* L.) plants under greenhouse and outdoor conditions. Additionally, we recommend that the biostimulating effect of CSL is further studied to determine its mechanisms of action on plant metabolism under abiotic stress.

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