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Integrative Effects of Zinc Nanoparticle and PGRs to Mitigate Salt Stress in Maize

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Abstract: Salinity is one of the most critical problems for agricultural development and threatens future food safety. Therefore, we aimed to investigate root application of zinc oxide nanoparticles (ZnO-NPs; 0, 50, 100 mg/L), 24-epibrassinolide (EBL; 0, 0.02, 0.04 µM), and their combinations on the growth and performance of maize (*Zea mays* L.) as a model plant grown under salt stress (i.e., 0, 5 and 10 dS m^{-1}) in a hydroponic system. The results showed that the highest salt stress negatively affected growth, physiological, and biochemical traits of maize. However, the application of EBL, ZnO-NPs, and their combinations significantly mitigated salt stress and improved the growth and performance of the physiological system in maize plants. In particular, the combination treatment of 100 mg/L ZnO-NPs + 0.02 µM EBL surpassed all other root treatments and resulted in the highest root and shoot growth, leaf area, relative leaf water content, net photosynthesis, total chlorophyll content, and uptake of zinc (Zn) and potassium (K). Furthermore, it minimized salt stress by reducing Na uptake, Na/K ratio, and proline in stressed maize plants. For example, the combination treatment of 100 mg/L ZnO-NPs + 0.02 µM EBL improved root length by +175%, shoot length by +39%, leaf area by +181%, RWC by +12%, net photosynthesis by +275, total chlorophyll content by +33%, and total phenolic content by +38%, in comparison to those obtained from the control, respectively. Furthermore, it enhanced the roots and leaves uptake of Zn under high salt stress treatment (i.e., 10 dS m^{-1}) by +125% and +94%, and K^+ by +39% and +51%, as compared to those grown without any of NPs or EBL treatments, respectively. Thus, the root application of 100 mg/L ZnO-NPs + 0.02 µM EBL can be a potential option to mitigate salt stress and improve the physiological, biochemical, and performance of strategy crops such maize.

Keywords: sustainable agriculture; ZnO-NPs; saline stress; EBL; strategies crops

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1. Introduction

The current global population growth rate is 1.1%, and it is predicted that the productivity of agriculture has to grow by 70–110% to ensure a sustainable and healthy food supply by the middle of the current century [1–3]. However, the production of major food crops has increased by 1% per annum on average [4]. In addition, climate change and anthropological activities are further deteriorating the limited agricultural resources [5]. Under such circumstances, the adoption of modern agricultural practices, such as Controlled Environment Agriculture (CEA), can possibly contribute towards self-sufficiency in food production [6,7]. Nevertheless, along with high development costs, energy inputs, high-tech mechanization, and elite knowledge, water salinity is one of the major limitations hindering CEA's wide adaptability at the farmer level [8].

Hydroponic techniques are currently gaining status as an efficient plant-growth system, especially under a controlled environment. Such plant factories have an advantage over conventional field farming as they can be separated from the natural environment and hence are less reliant on natural climatic conditions [6,8]. Recently, the opportunity of growing grain crops, such as wheat, rice and maize, has attained enough attention as a viable option

in countries with harsh climatic conditions [9,10]. However, the availability of good-quality water is one of the major limitations of hydroponics in water scarce countries. Thus, the use of saline water and treated wastewater is being investigated under different capacities by agricultural scientists to minimize the cost of desalination [11]. Recent studies have shown a significant negative impact of saline stress on plants grown under hydroponics, such as Biquinho' pepper (*Capsicum annuum* L.) [11], tomato (*Solanum lycopersicum* L.) [12–14], lettuce (*Lactuca sativa* L.) [15], and cucumber (*Cucumis sativus* L.) [16,17].

One of the most important technologies nowadays in mitigating abiotic stress is nanotechnology. The efficacy of nanoparticles as cost-effective, diverse, and highly efficient chemicals makes them revolutionary in the modernization of agriculture [18,19]. Zinc oxide nanoparticles (ZnO-NPs) have gained much attention in recent investigations, and can be used as nano-fertilizers and nano-growth regulators [20]. Although the mechanisms of their action in plants are yet not fully explored, they can enhance growth and yield of plants due to the efficient delivery of zinc (Zn) and are considered an essential plant micronutrient [21,22]. ZnO-NPs can improve seed germination, seedling development, photosynthetic activity, and Zn uptake. They can also modify protein synthesis in plants [21,23]. Recently, some studies have reported that ZnO-NPs can be one of the most important abiotic stress mitigators due to their key role in antioxidant biosynthesis, enzymatic activity, and carbohydrate metabolism in stressed plants [19,24–27]. However, the ZnO-NPs' toxicity and the negative effects on agricultural ecosystems cannot be overlooked. Thus, in order to optimize the dose and efficient methods of application to maximize the beneficial effects of ZnO-NPs in agriculture, it is of great significance to explore their effect on different plant traits in depth.

On the other hand, the application of plant growth regulators (PGRs) is one of the promising agronomic methods to mitigate the abiotic stresses in plants [28]. Brassinosteroids (BRs) are phytohormones that widely exist throughout the plant kingdom and play an important role in seed germination, seedling development, and plant growth. An active by-product from brassinolide biosynthesis is 24-epibrassinolide (EBL), which can mitigate the abiotic stresses in different plants [29–31]. It can improve the activity of photosynthetic enzymes in plants such as phosphoenol-pyruvate carboxylase (PEPcase), adenosine triphosphate synthetase (ATPase) and ribulose-1,5-bisphosphate carboxylase (RuBPase). Consequently, this can increase CO_2 fixation and photosynthesis efficiency [30,32,33]. Some recent studies have highlighted its role in abiotic stress amelioration [34], such as heat [35], chilling [36], drought [32,37], heavy metals [38,39], and salinity [40–43]. However, its role in mitigation of the saline stress especially under hydroponics is not well known.

The current study aimed to investigate the effects of root application of EBL and ZnO-NPs as saline stress mitigators on growth, biochemical, and physiological traits of maize (*Zea mays* L.) seedlings and mineral uptake as a model agronomic crop under semi controlled hydroponic glasshouse.

2. Materials and Methods

2.1. Growth Conditions, Experimental Design, and Plant Materials

Hybrid-310 maize seeds, with a 95% germination rate, were sown in agricultural perlite in vertical columns made of polyvinyl chloride (PVC). The pot height was 45 cm and its diameter was 9 cm. Five seeds were sown at sowing, then three seedlings per column were maintained after germination and the rest were removed. The research was led at a controlled hydroponic system, Glasshouse, College of Food and Agriculture Sciences, King Saud University, Saudi Arabia. The temperature was maintained at 28 ± 1 °C and 22 ± 1 °C for days and nights, respectively. A relative humidity of 60 ± 5%, along with 14 h of light and 10 h dark, was kept constant throughout the experiment. The factorial experiment was arranged in a completely randomized design (RCD) with three salt stress as a main factor (i.e., control, 5 dS m^{-1}, and 10 dS m^{-1}) and nine treatments of EBL, ZnO-NPs, and their combinations as sub-factor (i.e., control, EBR 0.02 µM, EBR 0.04 µM, ZnO-NPs 50.0 mg/L, ZnO-NPs 100.0 mg/L, EBR 0.02 µM + ZnO-NPs 50.0 mg/L, EBR

0.02 µM + ZnO-NPs 100.0 mg/L, EBR 0.04 µM + ZnO-NPs 50.0 mg/L, and EBR 0.04 µM + ZnO-NPs 100.0 mg/L). The EBL and ZNO-NPs were added in the nutrient solutions (i.e., hydroponic solution).

2.2. Nutrient Solution and Treatments

All experimental units were fed evenly with a hydroponic solution as follows: 0.5 µM potassium nitrate (KNO_3), 2.5 µM ammonium chloride (NH_4Cl), 0.5 µM magnesium sulfate ($MgSO_4$), 5.0 µM manganese monosulfate ($MnSO_4$), 2.5 µM monopotassium phosphate (KH_2PO_4), 30.0 µM boric acid (H_3BO_3), 100.0 µM ferric EDTA (Fe–K–EDTA), 1.0 µM ammonium heptamolybdate ($(NH4)_6Mo_7O_{24}$), 1.0 µM copper sulfate ($CuSO_4$), 0.5 µM calcium nitrate ($Ca(NO_3)_2$), and 1 µM zinc sulfate ($ZnSO_4$) [44]. The electrical conductivity (EC) of the solution was maintained at 1.1 dS m^{-1} and the pH was adjusted at 5.5.

2.3. Evaluation of the Seedling's Growth Parameters

Seven-week old plants were gently uprooted to measure plant growth traits, such as number of leaves per plant, stem diameter (mm), shoot length (cm), shoot fresh weight (g), leaf area (cm^2), and relative water content (%). The average stem diameter was measured at 5 cm above the first root using a Vernier caliper. However, the shoot length was measured using a metric scale. In addition, shoot dry weight and specific leaf weight were determined from oven-dried plant shoot samples for 72 h at 65 ± 1 °C. Leaf area was measured using LI-3000C leaf area meter (LI-COR, Lincoln, NE, USA), and the total plant leaf area was divided by the number of leaves to obtain the average leaf area. To determine the relative leaf water content (RWC), a disc-shaped (2 cm) piece of fresh leaf was dipped in distilled water for 12 h and then weighed to measure the turgor weight. The turgor samples were then placed in an electric oven at 65 ± 1 °C till constant weight, and RWC was calculated using Equation (1).

$$RWC = \frac{Turgor\ weight - Fresh\ weight}{Turgor\ weight - Oven\ dry\ weight} \times 100 \quad (1)$$

2.4. Measurement of the Root Parameters

After the plants were gently uprooted from the agricultural perlite, the roots were detached from the stem and washed with tap water. The fresh roots were then surface-dried using paper towels and weighed immediately, while the root length was measured with a meter scale. Root samples were then tainted with food color by submerging them in a colored solution for 24 h, then dried with paper towels and scanned using a root scanner. Then, WinRHIZO software (v5.0, Regent Instruments, Quebec, QC, Canada) was used for root photographs to analyze root traits such as total root length (cm), total surface area (cm^2), total number of root tips, average diameter (mm), and root volume (cm^3). Finally, the above mentioned root samples were dried in an electric oven at 65 °C for 72 h to get dry weight of roots.

2.5. Measurement of Photosynthetic Traits and Maximum Quantum Yield of PSII

Net photosynthesis, intercellular CO_2 concentration (Ci), and transpiration rate (Tr-mmol) were quantified using a portable LI-6400XT (LI-COR, Li-COR, Lincoln, NE, USA) at 25, 32, 39, and 46 days after sowing (DAS). The maximum quantum yield of PSII was recorded at 46 DAS using Handy PEA+ (Hansatech Instruments Ltd., Norfolk, UK); the plant leaves were dark adapted for 30 min using clips.

2.6. Determination of the Photosynthesis Pigments and SPAD

SPAD readings were recorded using a SPAD 502 Plus (Spectrum Technologies, Bridgend, UK) and the photosynthesis pigments, such as total chlorophyll and carotenoids, were analyzed at 46 DAS. Also, 5.00 g of fresh leaf samples were added to 10 mL of acetone solution (80%) and centrifuged (5000 rpm) for 5 min. The samples were then incubated in the

dark for 3 h before measuring the absorbance at 480, 510, 645, and 663 wavelengths [45,46] to calculate the total chlorophyll and carotenoids.

2.7. Na^+, K^+ and Zn^{2+} Concentration

Dry weight (0.5 g) from each replication of all treatments were ground and digested following Wolf's method [47], and the sample extracts were then analyzed using Flame photometer (Corning 400, Sherwood Scientific Ltd., Cambridge, UK) for Na^+ and K^+ content. In addition, the ICP-OES (PerkinElmer Optima 4300 DV ICP-OES, Waltham, MA, USA) was used to determine Zn^{2+} content in plant tissues.

2.8. Proline and Phenolic Content

To analyze proline content (mg/g FW), 5.00 g of fresh plant sample was homogenized in 10 mL of 3% sulfosalicylic acid. The resultant mixture was centrifuged for 10 min at 5000 rpm using Benchtop Centrifuge-5810R (Eppendorf, Hamburg, Germany), and then a 2 mL supernatant was extracted in a separate test tube. The glacial acetic acid and ninhydrin (2 mL each) were added to the extract (2 mL), and the mixture was incubated at 94–100 °C for 1 h in a boiling water bath. The samples were cooled. The chromophore containing toluene (4 mL) was collected in a separate tube after mixing for 20 s. The absorbance was analyzed at 520 nm using UV–VIS spectrophotometer (SHIMADZU, Kyoto, Japan, UV1800) [48], whereas the standard curve was obtained using known proline concentration.

To measure total phenolic content, 250 mg of each sample from the fresh leaves was ground as powder in liquid nitrogen followed by extraction with 10 mL of 80% methanol at constant temperature of 37 °C, as described by Tawaha et al. [49]. After three hours of shaking with methanol, the extract was cooled at 4 °C and centrifuged at 3500 rpm. The total phenolic content were estimated using Folin-Cicalteu colorimetric method [50].

2.9. Statistical Analysis

The data obtained from various analyses, such as roots, growth, physiological, and elemental analysis, were statistically processed for analysis of variance (ANOVA) using PASW statistics 21.0 (IBM Inc., Chicago, IL, USA). Treatment means were compared using Duncan multiple range test for the significant differences at $p \leq 0.05$.

3. Results and Discussion

3.1. Root Growth and Development

The effect of saline stress, the main factor, stood highly significant for all studied parameters, where the highest saline stress (10 dS m^{-1}) reduced the total root length, total surface area, average diameter, root total volume, and number of root tips by −22%, −31%, −29%, −30%, and −29%, respectively, as compared to unstressed plants (Table 1). However, the root application of ZnO-NPs and EBL, either alone or as a combination of their various concentrations, improved root growth and development by minimizing the deadly effects of saline stress. Treatment 7 (ZnO-NPs at 100 mg/L and EBL at 0.02 µM) showed significantly higher results by improving the total root length, surface area, average diameter, root total volume, and number of root tips by +175%, +157%, +75%, +261%, and +159%, respectively, as compared to unstressed plants (Table 1). Concerning the interaction effects, the shortest total root lengths (173.29, 235.30, 283.29, and 304.28 cm) were recorded for T1, T2, T4, and T3 when plants were grown with high saline stress (10 dS m^{-1}), whereas the highest values (744.46, 676.89, and 607.78 cm with T7) were recorded when plants were exposed to 0, 5, and 10 dS m^{-1} saline stress, respectively. In addition, the application of T7 and T9 resulted in the highest values of average diameter, surface area, number of root tips, and total volume under different saline stress treatments. For example, the root surface area was reduced by −51, −39, and −8% with treatments of T1, T2, and T4 under 10 dS m^{-1} saline stress, while it was increased by +103 and +93% with treatments of T7 and T9 under 5 dS m^{-1} saline stress in comparison to those obtained from the control (0 dS m^{-1} + T1), respectively (Table 1).

Table 1. Effect of ZnO-NPs and EBL on the number of root tips, total root volume, average diameter, total surface area, and root length of maize plants under saline stress.

Salinity	Root Application Treatments	Total Root Length (cm)	Total Surface Area (cm^2)	Average Diameter (mm)	Total Root Volume (cm^3)	Number of Root Tips (Number)
Control	T1	296.90	256.91	1.84	10.96	1237.33
	T2	326.38	291.12	1.94	12.89	1341.33
	T3	421.72	320.55	2.11	16.92	1681.33
	T4	365.01	310.44	1.82	15.40	1504.33
	T5	443.80	343.66	2.25	17.46	1788.67
	T6	456.95	377.89	2.47	28.41	2061.67
	T7	744.46	605.64	3.03	37.02	2875.00
	T8	531.32	482.50	2.53	31.90	2734.00
	T9	617.61	582.55	2.77	30.02	2680.00
	Mean	**467.13 A**	**396.81 A**	**2.31 A**	**22.33 A**	**1989.30 A**
5 dS m^{-1}	T1	267.45	238.95	1.62	9.71	987.67
	T2	294.13	258.94	1.77	11.16	1107.33
	T3	367.04	300.16	1.82	14.96	1411.00
	T4	340.61	270.88	1.56	13.74	1239.33
	T5	403.98	316.25	1.96	14.72	1537.67
	T6	421.57	341.63	2.07	23.48	1781.33
	T7	676.89	521.96	2.62	33.19	2601.00
	T8	490.44	407.14	2.18	26.51	2373.00
	T9	565.68	495.65	2.23	27.03	2225.33
	Mean	**425.31 B**	**350.17 B**	**1.98 B**	**19.39 B**	**1695.96 B**
10 dS m^{-1}	T1	173.29	125.80	1.13	6.66	787.67
	T2	235.30	157.41	1.37	9.68	914.00
	T3	304.28	244.85	1.56	12.26	1168.00
	T4	283.29	235.25	1.28	11.54	1025.33
	T5	377.46	268.16	1.67	12.82	1366.67
	T6	378.36	296.04	1.64	18.71	1457.00
	T7	607.78	469.62	2.35	28.65	2340.00
	T8	448.04	323.14	1.78	20.72	1954.67
	T9	450.56	342.41	1.89	18.75	1740.00
	Mean	**362.04 C**	**273.63 C**	**1.63 C**	**15.53 C**	**1417.04 C**
Significance ANOVA						
Saline Stress		***	**	***	***	***
Seed Priming Treatment		***	***	**	**	*
Interaction		***	**	*	***	**
$SEM_{0.05}$						
Saline Stress		3.040	3.279	0.017	0.161	12.969
Seed Priming Treatment		5.265	5.679	0.029	0.279	22.462
Interaction		9.119	9.837	0.051	0.483	38.906

T1 = control, T2 = EBR 0.02 µM, T3 = EBR 0.04 µM, T4 = ZnO-NPs 50.0 mg/L, T5 = ZnO-NPs 100.0 mg/L, T6 = EBR 0.02 µM + ZnO-NPs 50.0 mg/L, T7 = EBR 0.02 µM + ZnO-NPs 100.0 mg/L, T8 = EBR 0.04 µM + ZnO-NPs 50.0 mg/L, and T9 = EBR 0.04 µM + ZnO-NPs 100.0 mg/L. *** = $p \leq 0.001$; ** = $p \leq 0.01$; * = $p \leq 0.05$. SEM = Standard Error of Means. Capital letters show the significance differences among salt stress treatments.

A plant's roots are the primary organs directly exposed to saline stress, which can be further divided into osmotic stress and ionic toxicity [51]. Salinity creates a hypertonic

microenvironment in the soil, which disrupts the osmotic balance between the extracellular solution and cell sap of the root cells [52]. Thus, the negative water potential leads to physiological drought, limiting the water availability and nutrient uptake by root cells [53]. Consequently, impaired cell division, cell elongation, and cell growth limit the root growth in all dimensions, such as length, diameter, and volume. Na^+ and Cl^- entered in cytoplasm cannot be sequestrated by tonoplast, and thus, the over accumulation of these ions leads to ionic toxicity in root cells [51]. In response to saline stress, the over synthesis of reactive oxygen species (ROS) elevates lipid peroxidation and lipoxygenase acidity, which decomposes phospholipids of cellular membranes [54]. Moreover, in addition to the denaturation of cellular structural proteins, Na^+ toxicity directly disrupts the cell cycle, mitotic division, and cellular metabolism in meristematic tissues, thus retarding root growth, development, and morphological features [51,52,55,56].

The results of the current study highlighted significant enhancement in root growth characteristics in response to EBL and ZnO-NPs treatments even under saline stress conditions (Table 1). Zinc, being the cofactor of several key enzymes, plays an imperative role in cellular metabolism and homeostasis, especially under abiotic stresses, such as salinity. Upon binding with phospholipids and sulfhydryl groups, Zn ameliorates stress induced denaturation of cellular structures and hence regulates its stability and functionality [57,58]. Furthermore, Zn supports the biosynthesis of auxin and hence promotes cell division and elongation, which results in high biomass production and hence root growth [59]. EBL as a master hormone can play a key role in the cell cycle and mitotic division, thus promoting cell elongation and proliferation in root meristematic tissues, which could potentially alleviate the deleterious effects of Na^+ toxicity in meristematic cells and facilitate root growth and development [60]. Sousa et al. [42] reported a 1.19-fold increase in root diameter and 12% thicker epidermis when *Solanum lycopersicum* L. plants were pretreated with 100 nM EBL solution. Thus, our findings proved the use of EBL and ZnO-NPs as abiotic stress mitigators and could be used in hydroponics to enhance tolerance in plants against saline stress.

3.2. Seedling Growth Performance

The results obtained showed that the impact of the main factor, saline stress, stood highly significant for all studied seedling growth parameters. High saline stress (10 dS m^{-1}) caused −38%, −38%, −20%, −36%, and −11% decline in the stem diameter, shoot length, specific leaf weight (SLW), leaf area, and relative water content (RWC), respectively, as compared to the control (Table 2). Nevertheless, the root application of EBL and ZnO-NPs, either alone or as combination of their various concentrations, ameliorated the deleterious effects of saline stress on seedling growth. The combined application of EBL at 0.02 µM and ZnO-NPs at 100 mg/L (T7) resulted in a significantly higher improvement in stem diameter, shoot length, leaf area, and RWC, as +31%, +39%, +181%, and +12%, respectively (Table 2). A highly significant interaction between the root application of EBL and ZnO-NPs treatments and saline stress was found for the aforesaid seedling growth characteristics. The saline stress as 5 dS m^{-1}(medium) and 10 dS m^{-1} (high) with T1 (control) resulted in 9.00 mm and 5.10 mm stem diameter, respectively. Whereas T7 improved the stem diameter by +14% and +54% compared to the respective controls (T1) in medium and high saline stress, respectively. The shortest shoot lengths (50.93, 57.43, and 60.50 cm) were measured in T1, T4, and T5, respectively, when maize seedlings had grown under 10 dS m^{-1} saline stress. However, T8, T9, and T7 enhanced the shoot length by +74%, +57%, and +57%, respectively, as compared to the control (T1) under high salinity of 10 dS m^{-1}. Similar trends were also seen for SLW and leaf area, where the root application of EBL and ZnO-NPs combinations (T6, T7, T8, and T9) minimized the saline induced decline and significantly improved the recorded values. Saline stress as 5 dS m^{-1} (medium) and 10 dS m^{-1} (high), as compared to the control under no stress, decreased the RWC by −5% and −19%, respectively, whereas T7 maintained RWC even higher than the control as +5% and +1% under medium and high saline stress, respectively (Table 3).

Table 2. Effect of EBL and ZnO-NPs application on stem diameter, shoot length, specific leaf weight (SLW), leaf area, and RWC of saline-stressed maize plants.

Salinity	Root Application Treatments	Stem Diameter (mm)	Shoot Length (cm)	SLW (mg/cm^2)	Leaf Area (cm^2)	RWC (%)
Control	T1	9.77	98.90	3.55	80.46	86.23
	T2	8.80	105.67	2.80	107.33	87.48
	T3	8.10	99.77	2.95	118.64	88.98
	T4	11.03	103.07	3.44	153.81	87.12
	T5	12.27	108.40	3.26	188.35	88.29
	T6	12.27	118.87	3.45	182.20	90.05
	T7	13.07	126.33	3.04	233.06	90.55
	T8	12.57	121.60	3.38	202.46	89.22
	T9	10.03	115.03	2.66	212.88	90.04
	Mean	**10.88 A**	**110.85 A**	**3.17 A**	**164.35 A**	**88.66 A**
5 dS m^{-1}	T1	9.00	70.60	3.70	94.64	82.14
	T2	8.47	81.70	1.99	172.51	83.89
	T3	8.57	88.07	1.85	142.00	85.18
	T4	10.23	94.47	3.47	128.15	87.07
	T5	10.43	84.73	2.32	155.81	89.41
	T6	9.57	81.83	2.90	174.21	88.27
	T7	10.27	100.10	3.08	206.80	90.12
	T8	9.50	85.70	2.79	187.54	88.05
	T9	9.13	85.03	2.92	138.59	89.69
	Mean	**9.46 B**	**85.80 B**	**2.78 B**	**155.58 B**	**87.09 B**
10 dS m^{-1}	T1	5.10	50.93	2.69	34.47	69.97
	T2	5.07	67.43	2.02	64.35	73.66
	T3	7.03	62.13	2.49	66.68	72.44
	T4	7.23	57.43	2.72	74.04	77.16
	T5	7.30	60.50	2.00	105.08	78.62
	T6	7.53	77.63	3.09	137.13	82.62
	T7	7.87	80.03	3.25	148.57	87.37
	T8	7.23	88.53	2.78	156.37	86.16
	T9	6.57	80.30	1.69	152.81	84.78
	Mean	**6.77 C**	**69.44 C**	**2.53 C**	**104.39 C**	**79.20 C**
Significance ANOVA						
Saline Stress		**	***	***	***	**
Seed Priming		***	**	**	**	***
Interaction		***	***	*	***	**
SEM$_{0.05}$						
Saline Stress		0.093	0.574	0.045	2.537	0.231
Seed Priming Treatment		0.161	0.994	0.077	4.395	0.399
Interaction		0.278	1.721	0.134	7.612	0.692

T1 = control, T2 = EBR 0.02 µM, T3 = EBR 0.04 µM, T4 = ZnO-NPs 50.0 mg/L, T5 = ZnO-NPs 100.0 mg/L, T6 = EBR 0.02 µM + ZnO-NPs 50.0 mg/L, T7 = EBR 0.02 µM + ZnO-NPs 100.0 mg/L, T8 = EBR 0.04 µM + ZnO-NPs 50.0 mg/L, and T9 = EBR 0.04 µM + ZnO-NPs 100.0 mg/L. *** = $p \leq 0.001$; ** = $p \leq 0.01$; * = $p \leq 0.05$. SEM = Standard Error of Means. Capital letters show the significance differences among salt stress treatments.

Table 3. Effect of EBL and ZnO-NPs application on total chlorophyll, carotenoids, phenolic content, and proline of saline stressed maize plants.

Salinity	NPs/EBL	Total Chlorophyll (mg/gFW)	Carotenoids (mg/gFW)	Phenolic Content (mgGAE/gFM)	Proline (mg/gFW)
Control	T1	4.53	0.35	40.25	0.37
	T2	4.57	0.36	41.56	0.37
	T3	4.89	0.38	40.99	0.37
	T4	4.77	0.37	43.04	0.37
	T5	4.95	0.39	45.44	0.36
	T6	4.89	0.37	43.11	0.36
	T7	5.18	0.40	45.85	0.34
	T8	4.97	0.38	42.59	0.36
	T9	5.00	0.37	42.78	0.36
	Mean	**4.86 A**	**0.37 A**	**42.85 B**	**0.36 C**
5 dS m^{-1}	T1	3.79	0.29	47.28	2.05
	T2	4.29	0.31	50.91	1.35
	T3	4.61	0.36	54.61	0.76
	T4	4.68	0.34	55.87	0.85
	T5	4.80	0.36	64.13	0.70
	T6	4.63	0.35	56.87	0.67
	T7	5.03	0.38	69.71	0.62
	T8	4.83	0.35	66.23	0.71
	T9	4.76	0.34	66.82	0.76
	Mean	**4.60 B**	**0.34 B**	**59.16 A**	**0.94 B**
10 dS m^{-1}	T1	3.03	0.21	24.33	2.60
	T2	3.80	0.28	28.58	1.67
	T3	4.44	0.31	32.36	1.19
	T4	4.14	0.30	35.15	0.95
	T5	4.50	0.32	36.17	0.82
	T6	4.11	0.29	35.79	0.75
	T7	4.84	0.34	41.27	0.69
	T8	4.54	0.31	35.20	0.75
	T9	4.28	0.31	37.37	0.73
	Mean	**4.19 C**	**0.30 C**	**34.03 C**	**1.13 A**
Significance ANOVA					
Saline Stress		**	***	***	***
Seed Priming		***	**	***	***
Interaction		*	**	***	**
$SEM_{0.05}$					
Saline Stress		0.014	0.001	0.297	0.007
Seed Priming		0.024	0.002	0.514	0.013
Interaction		0.042	0.004	0.890	0.022

T1 = control, T2 = EBR 0.02 μM, T3 = EBR 0.04 μM, T4 = ZnO-NPs 50.0 mg/L, T5 = ZnO-NPs 100.0 mg/L, T6 = EBR 0.02 μM + ZnO-NPs 50.0 mg/L, T7 = EBR 0.02 μM + ZnO-NPs 100.0 mg/L, T8 = EBR 0.04 μM + ZnO-NPs 50.0 mg/L, and T9 = EBR 0.04 μM + ZnO-NPs 100.0 mg/L. *** = $p \leq 0.001$; ** = $p \leq 0.01$; * = $p \leq 0.05$. SEM = Standard Error of Means. Capital letters show the significance differences among salt stress treatments.

The root cells actively participate in controlling Na^+ entry in the transpiration stream to minimize the ionic toxicity in aerial parts of the stressed plants [56]. However, prolonged saline stress accumulates Na^+ and Cl^- ions in various cellular components in leaves up to noxious level, which subsequently interfere metabolic activities, physiological processes, protein synthesis, and cell homeostasis [52,61]. Moreover, the overrun of ROS leads to oxidative damage of cell structures and nucleus acid, which could imbalance cell functions and even cause cell death [56,62]. The saline stress induced denaturation of cell membrane results in electrolyte leakage and may affect turgor pressure, cell elongation, and cell multiplication. The counter mechanism, biosynthesis of organic electrolytes to complete Na^+, poses an additional pressure on cell metabolism and hence can affect carbon partitioning and ultimately the plant growth. Furthermore, the widened Na^+/K^+ disturbs stomatal conductance, leading to the impairment of photosynthesis which consequently limits plant growth and growth associated traits [63,64].

ZnO-NPs provide a slow but continuous supply of Zn^{2+}, which promotes the production of endogenous plant regulators (i.e., gibberellic acid (GA3) and indole-3-acetic acid (IAA)) and accelerates plant growth by maintaining cell membrane stability, cell division, and enzymatic activity [65,66]. In spinach (*Spinacia oleracea*), the foliar application of ZnO-NPs under saline stress resulted in an enhanced biosynthesis of the photosynthetic pigments, which caused high shoot length and dry weight [67]. Phytochromes, such as EBL, are of significant importance for their role in the cell cycle, cell differentiation, and elongation [68]. Nolan et al. [69] conveyed that EBL promotes xylem regeneration and cell elongation, which resulted in higher plant growth and biomass under saline stress conditions in comparison with the control. In *Oryza sativa* L., the exogenous application of EBL significantly improved both vegetative growth and net dry mass in plants under 200 mM NaCl stress [38]. Similarly, in *Triticum aestivum* L., EBL foliar application by amelioration resulted in improved plant height, biomass production, and grain yield even in saline-stressed plants [70]. Desoky et al. [71] found that exogenous EBL application regulated osmotic adjustments at cellular level and restored RWC efficiently in plants under saline stress.

3.3. Photosynthesis and Contributing Traits

Photosynthesis and related physiological traits exhibited a substantial but detrimental effect of saline stress; however, the application of various concentrations of EBL and ZnO-NPs, either alone or in combination, significantly ameliorated the stress induced decline in study parameters (Figures 1–5). High (10 dS m^{-1}) saline stress consistently maintained a reduced rate of photosynthesis as −20%, −16%, −20%, and −20%, as compared to the control for 25, 32, 39, and 46 DAS (Figure 1). Furthermore, it was also observed that intercellular CO_2 concentration (Ci) as compared to the control was declined by −21%, −16%, −20%, and −21% for 25, 32, 39, and 46 DAS, respectively, when exposed to 10 dS m^{-1} (high) saline stress (Figure 2). The saline stress mediated reduction in transpiration rate, however, kept increasing gradually from −14% to −25% between 25 and 46 DAS under 10 dS m^{-1} (Figure 3). Leaf green index (LGI) and maximal photochemical efficiency of PSII (Fv/Fm) recorded at 46 DAS showed maximum deterioration of −34% and −12%, respectively, in maize seedlings grown under high (10 dS m^{-1}) saline stress (Figures 4 and 5).

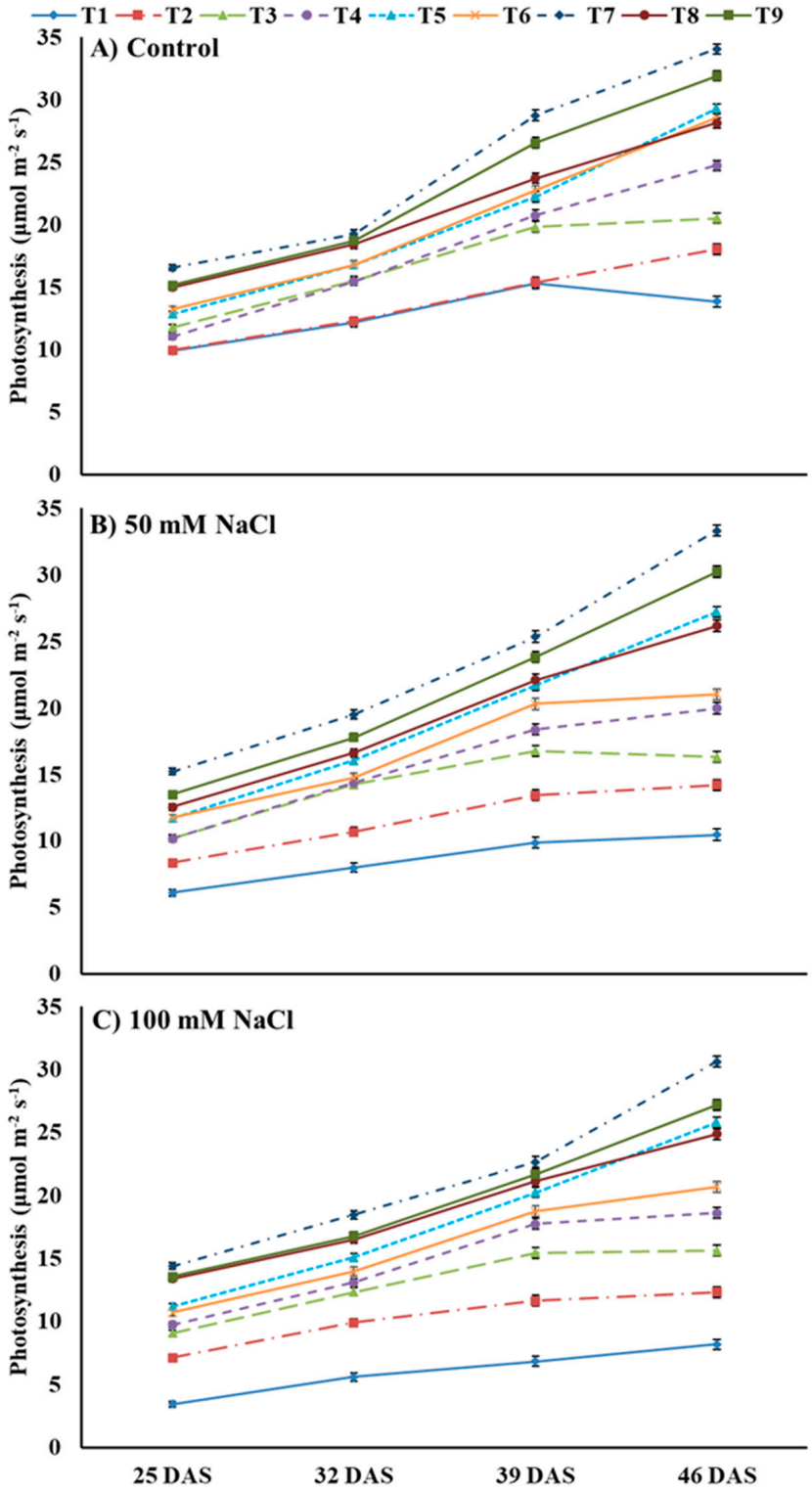

Figure 1. Effect salinity and root application of EBL and ZnO-NPs on the rate of photosynthesis of maize. Error bars represents square error mean (SEM at 0.05). T1 = control, T2 = EBR 0.02 µM, T3 = EBR 0.04 µM, T4 = ZnO-NPs 50.0 mg/L, T5 = ZnO-NPs 100.0 mg/L, T6 = EBR 0.02 µM + ZnO-NPs 50.0 mg/L, T7 = EBR 0.02 µM + ZnO-NPs 100.0 mg/L, T8 = EBR 0.04 µM + ZnO-NPs 50.0 mg/L, and T9 = EBR 0.04 µM + ZnO-NPs 100.0 mg/L. DAS = Day after sowing.

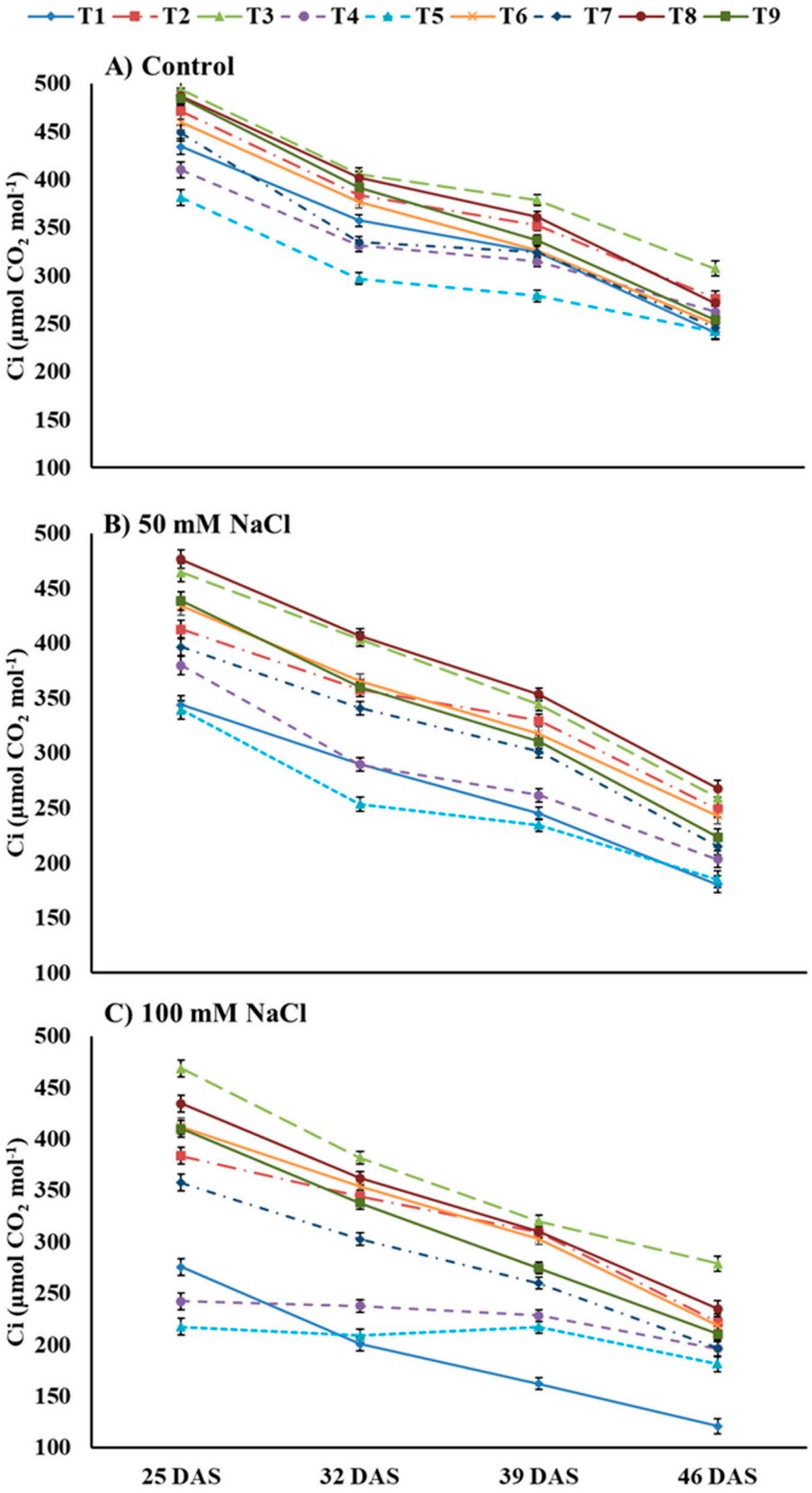

Figure 2. Effect salinity and root application of EBL and ZnO-NPs on the intercellular CO_2 concentration (Ci) of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

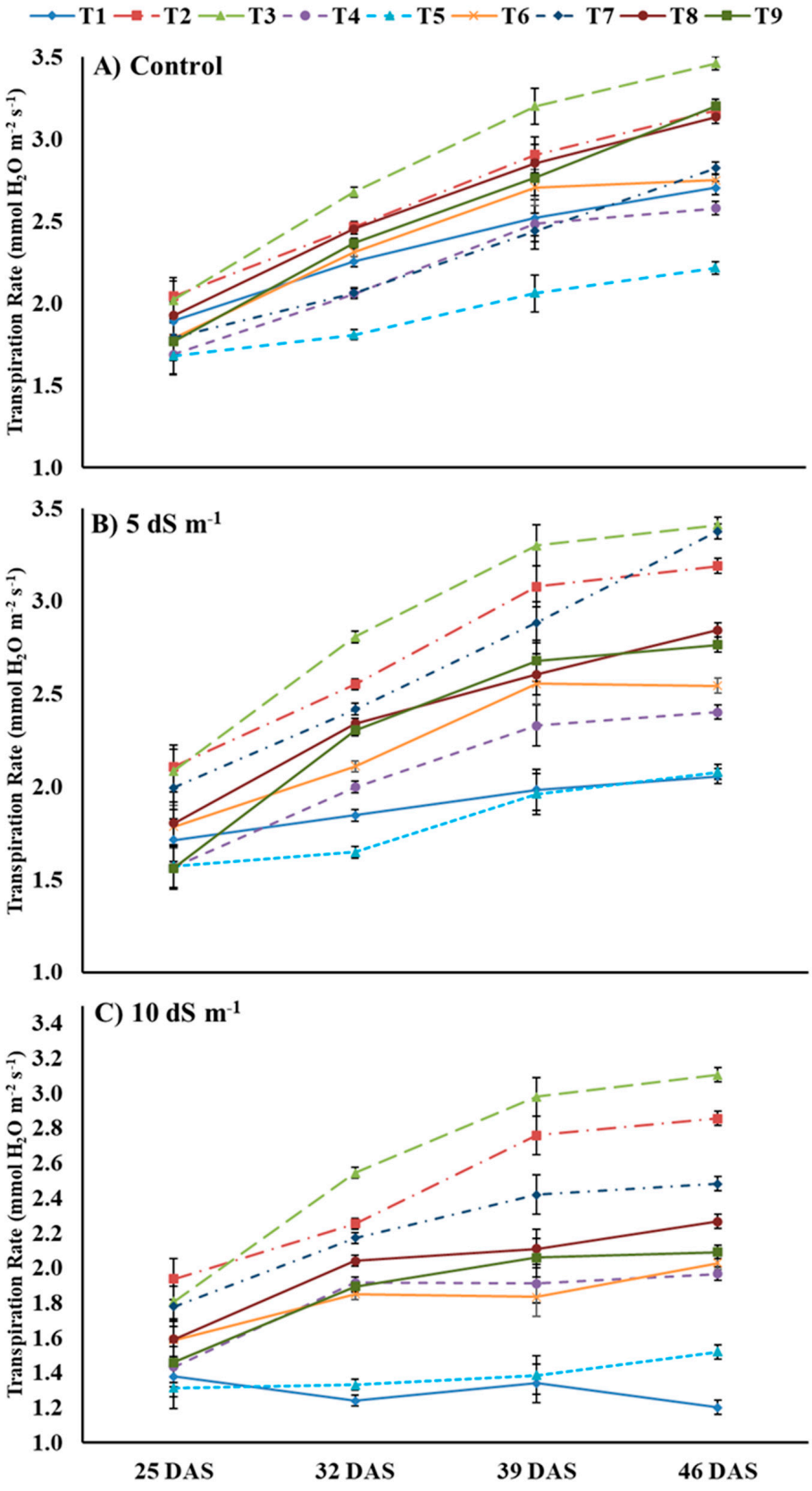

Figure 3. Effect salinity and root application of EBL and ZnO-NPs on the transpiration rate of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

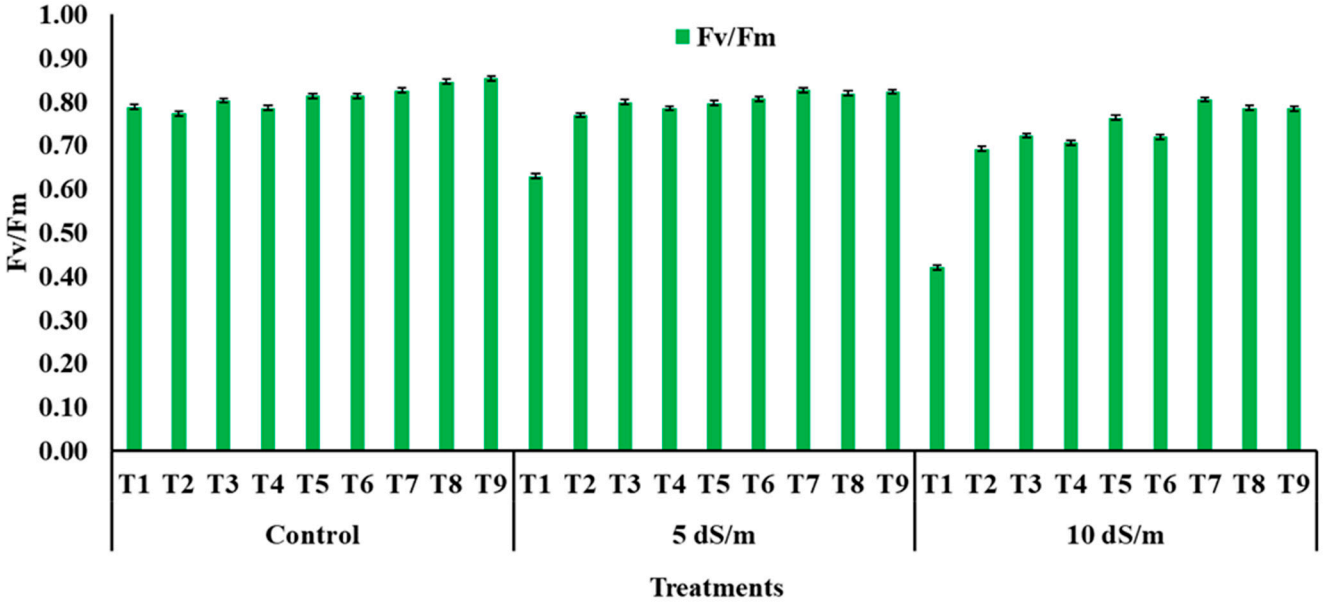

Figure 4. Effect salinity and root application of EBL and ZnO-NPs on the maximal photochemical efficiency of PSII (Fv/Fm) of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

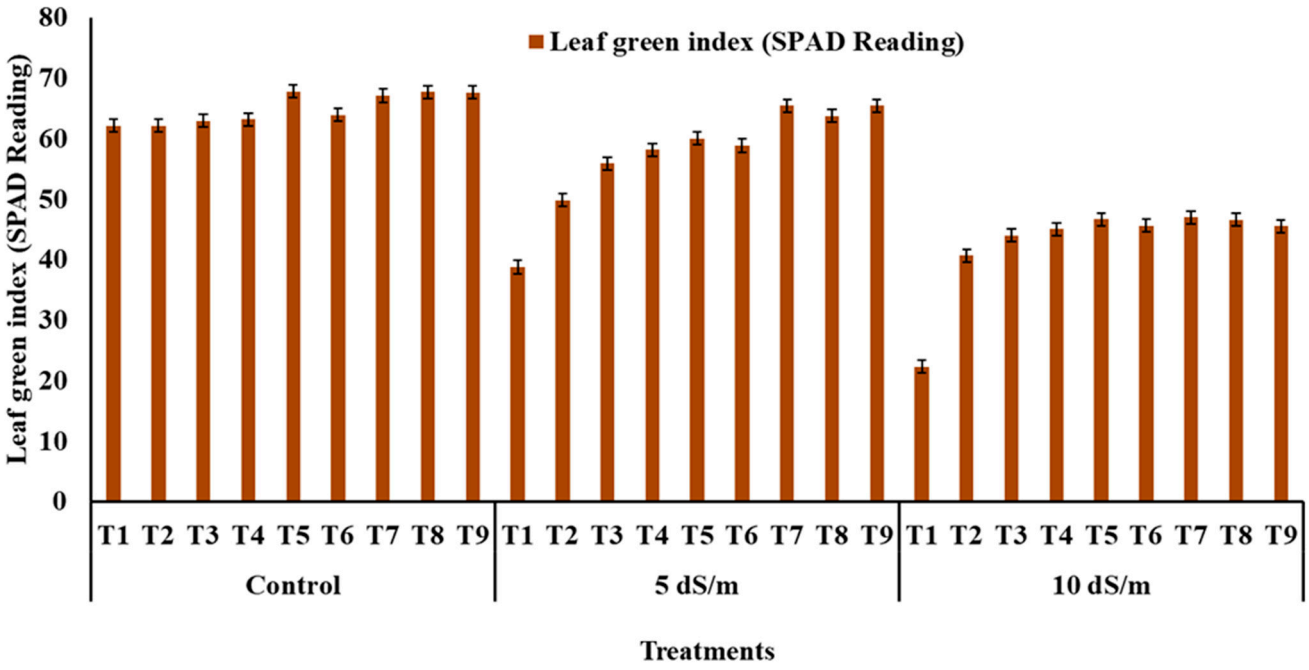

Figure 5. Effect salinity and root application of EBL and ZnO-NPs on the leaf green index (SPAD-reading) of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

Our results showed that photosynthesis and its associated parameters were significantly improved in response to root application of EBL and ZnO-NPs treatments (Figures 1–5). The combination of EBL at 0.02 µM and ZnO-NPs at 100 mg/L (T7) significantly and consistently resulted in the highest rate of photosynthesis under all saline stress conditions, i.e., +146%, +219%, and +275% higher than the control at 46 DAS for the control (0 dS m^{-1}), medium (5 dS m^{-1}) and high (10 dS m^{-1}) saline stress, respectively. Whereas T1 (control) stood the lowest among all treatments (Figure 1) throughout the plant growth

period. EBL alone at 0.04 µM (T3) resulted in highest intercellular CO_2 concentration under high (10 dS m^{-1}) saline stress, whereas T1 (control) once again was recorded for the lowest values (Figure 2). The transpiration rate showed that T3 (EBR at 0.04 µM) was consistently and significantly higher than all other treatments. It resulted in +28%, +66%, and +158% higher than the control (T1) at 46 DAS for 0, 5 and 10 dS m^{-1} saline stress, respectively (Figure 3). However, for (LGI) and maximal photochemical efficiency of PSII (Fv/Fm), the combined treatments of EBL and ZnO-NPs, such as T7, T8, and T9, stood statistically at par but significantly higher than all other treatments under medium and high saline stress, whereas the control (T1) resulted in lowest values when measured at 46 DAS (Figures 4 and 5).

Excessive accumulation of Na^+ in chloroplasts damages the photosynthetic apparatus, such as thylakoid membrane, electron transport chain, and the photosystem II complex, which consequently lowers the photosynthetic efficiency in stressed plants [52]. Yue et al. [72] reported denatured thylakoid membrane and swollen chloroplasts in *R. pseudoacacia* L. when plants were treated with saline stress for two weeks. Furthermore, Na^+ toxicity induces photo-inhibition by over-excitation of electrons, and results in reduced non-photochemical quenching (NPQ) [73]. Moreover, the saline stress mediated osmotic imbalance in leaf cells and stomatal closure induced by lower K^+ uptake adversity affects the transpiration and limits photosynthetic efficacy significantly [64,74].

The exogenous application of ZnO-NPs simulates biosynthesis of photosynthetic pigments, i.e., chlorophylls and carotenoids, under abiotic stresses [58]. Rizwan et al. [75] reported that ZnO-NPs foliar application alleviated saline stress induced oxidative damage of the photosynthetic apparatus and improved transpiration and net photosynthetic rates in *Triticum aestivum* L. Faizan et al. [39] reported significant improvement in photochemical efficiency of PS II, chloroplast structure, and rate of photosynthesis in *Oryza sativa* L. under saline stress when treated with ZnO-NPs. In *Triticum aestivum* L., the ZnO-NPs seed priming ameliorated Na^+ mediated modifications in the leaf proteins' electrophoretic profile and promoted chlorophyll biosynthesis and leaf infrastructures [76]. Similarly, in spinach seed priming with ZnO-NPs elevated carotenoids, chlorophyll a and b content in plants under saline stress [67]. The improved photosynthetic efficiency and physiological performance under saline stress caused by EBL root application in this study could possibly be accredited to the role of EBL as a master hormone. Through biochemical cross-talks, EBL regulates the endogenous synthesis, gene expression, and receptor mediated response of other plant hormones/regulators and hence controls a number of physiological activities [69]. Shahzad et al. [38] described that EBL exogenous application under saline stress minimized the Na^+ toxicity and structural damage of chloroplast and thylakoid membrane, which improved the photochemical efficiency of PS II and consequently resulted in higher photosynthetic activity, transpiration, and stomatal conductance in *Oryza sativa* L. In *Robinia pseudoacacia* L. under 100 mM NaCl stress, EBL remarkably elevated stomatal and mesophyll conductance reduced intercellular CO_2 concentration [72]. The improved photosynthetic activity in response to EBL can be associated with enhanced NPQ, Fv/Fm, electron transport chain, and biosynthesis of photosynthetic pigments [38,72].

3.4. Photosynthetic Pigments, Phenolic, and Proline Content

Photosynthetic pigments, such as chlorophyll and carotenoids, in maize seedlings showed significant reduction under saline stress conditions as compared to unstressed, i.e., 10 dS m^{-1} (high saline stress) caused −14%, −19%, and −21% reduction in total chlorophyll content, carotenoids, and total phenolic content, respectively (Table 3). However, the proline content were increased by +214% under high as compared to unstressed maize plants (Table 3). The root application of EBL and ZnO-NPs, either alone or as combined treatments of their different concentrations, significantly ameliorated the saline stressed induced reduction in photosynthetic pigments and phenolic content. A combination of EBL at 0.02 µM and ZnO-NPs at 100 mg/L (T7) improved total chlorophyll, carotenoids, and total phenolic content by +33%, +38%, and +40%, respectively, as compared to the control

(T1) (Table 3). Furthermore, T7 showed a significant reduction (−67%) in proline content as compared to T1 (Table 3). The interactive effect of saline stress and the application of various treatments of EBL and ZnO-NPs was also recorded as highly significant. As compared to the control (4.53 mg/gFW), the lowest total chlorophyll content (3.03, 3.79, and 3.80 mg/g FW) were found for T1 with 10 dS m^{-1}, T1 with 5 dS m^{-1}, and T2 with 10 dS m^{-1} saline stress, respectively. Whereas T7 improved the total chlorophyll content by +11% and +7% under medium (5 dS m^{-1}) and high (10 dS m^{-1}) saline stress in comparison with the control, respectively. Nonetheless, high saline stress (10 dS m^{-1}) in the absence of EBL and/or ZnO-NPs treatment lowered the carotenoids to 0.21 mg/g FW, whereas T7 and T5 improved by +62% and +52%, respectively. Similarly, T7 and T9 improved total phenolic content under 10 dS m^{-1} saline stress by +70% and +54%, respectively, as compared to T1 (control). Proline content, however, have shown the opposite trend, where the control (T1 + 0 dS m^{-1}) resulted in the lowest value (0.37 mg/gFW), whereas T1 under high (10 dS m^{-1}) saline stress produced maximum proline concentration (0.69 mg/gFW) but T7 and T9 succeeded to lower the proline content by 3.75 and 3.56 times as compared to T1 (Table 3).

Zinc is a well-known essential micronutrient which plays vital role in biosynthesis of photosynthetic pigments, such as chlorophyll, carotenoids and xanthophyll [77]. Raliya et al. [78] stated an increase in chlorophyll content of *Solanum lycopersicum* L. when ZnO-NPs (750 mgL^{-1}) was applied as foliar spray. Rai-Kalal and Jajoo [79] found significant improvement in chlorophyll a and b, carotenoids, and total phenolic content when seeds of *Triticum aestivum* L. were primed with 10 mgL^{-1} ZnO-NPs. Another study conducted on *Triticum aestivum* L. and *Zea mays* L. seed soaking with 200 mgL^{-1} of ZnO-NPs improved total chlorophyll and carotenoids in both crops by >50% [80]. Wu et al. [81] found that seed priming with 100 mgL^{-1} ZnO-NPs under arsenic (As) stress in *Oryza sativa* L. improved chlorophyll content by up to 40.1%. However, Wang et al. [82] reported that ZnO-NPs application beyond 300 mgL^{-1} in *Arabidopsis* showed toxic effects and reduced the chlorophyll (a and b) content > 50% as compared to the control, which consequently led to >50% decline in photosynthetic activity.

Brassinosteroids, such as EBL, potentially promote chlorophyll biosynthesis by regulating the chlorphyllase enzymatic activity under various abiotic stresses [34]. Sousa et al. [42] found that pretreatment with 100 nM EBL improved the photopigment concentration in plants under 150 mM NaCl stress in *Solanum lycopersicum* L. Kohli et al. [83] documented that EBL seed priming in *Brassica juncea* L. resulted in elevated total acrophyll, carotenoids, and total phenolic content in plants under Pb stress. Hosseinpour et al. [32] reported that 0.1 µM EBL foliar application in *Echinacea purpurea* L. elevated antioxidant production, total protein content, and proline under drought stress. Furthermore, Li et al. [43] highlighted enhanced proline, total chlorophyll content, and correlated photosynthetic activity in *Arachis hypogaea* L. under 150 mM NaCl stress when treated with 0.1 µM EBL foliar application.

3.5. Na, K, and Zn Uptake

Saline stress affected the uptake of Na, K, and Zn in maize plants grown under different treatments of EBL and ZnO NPs (Figures 6–8). It has significantly impacted the mineral profile of stressed plants, i.e., high (10 dS m^{-1}) saline stress reduced Zn^{2+} content in both roots and leaves by −29% and −42%, respectively, as compared to unstressed plants (Figure 6). Similarly, 10 dS m^{-1} saline stress as compared to unstressed (control) elevated the Na^+ content for both roots and leaves as +258% and +328%, respectively (Figure 7). Furthermore, the aforesaid saline stress caused a +405% and +494% increase in Na^+/K^+ as compared to the control (Figure 9). However, saline stress mediated response resulted in a −26% and −25% decline K^+ concentration, in both roots and leaves, respectively, under high (10 dS m^{-1}) saline stress (Figure 8). The combination of 0.02 µM EBL and 100 mg/L ZnO-NPs (T7) stood better than all other root treatments by buffering the toxic effects of induced salinity. The aforesaid treatment (T7) enhanced Zn^{2+} uptake and K^+ by +125% and +39%, and +94% and +51% in roots and leaves, respectively, in contrast with T1

(Figures 6 and 8). Furthermore, as compared to the control T7, it also reduced Na^+ content by −42% and −40%, and Na^+/K^+ by −50% and −53% in roots and leaves, respectively (Figures 7 and 9).

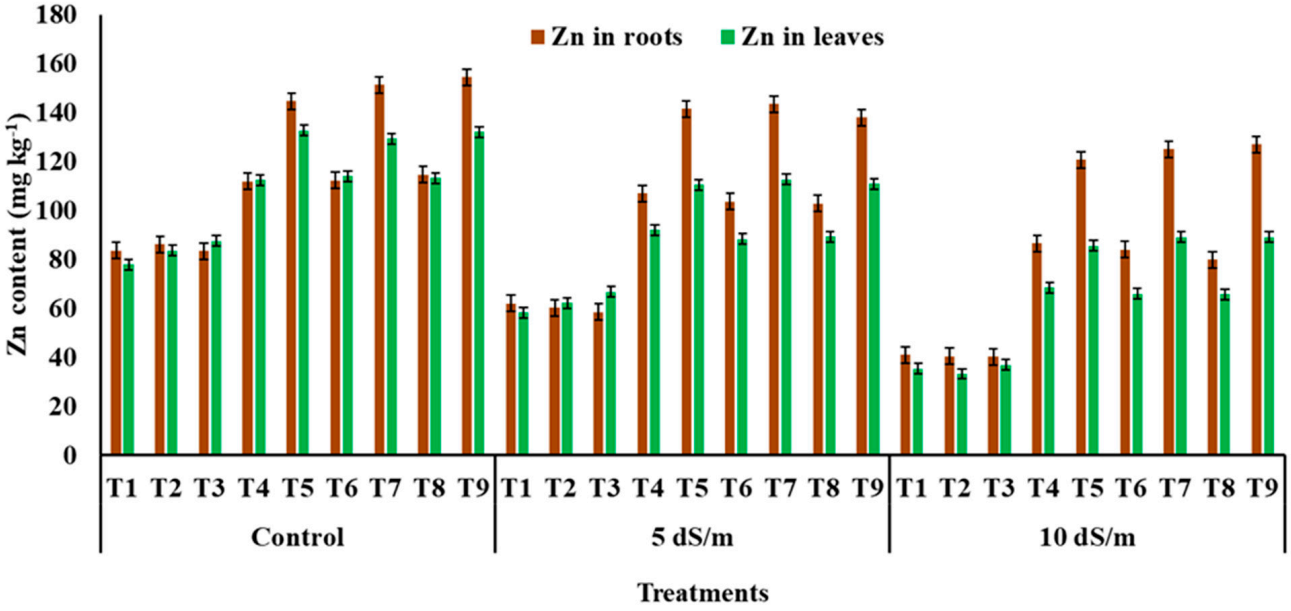

Figure 6. Effect salinity and root application of EBL and ZnO-NPs on the Zn content in both roots and leaves of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

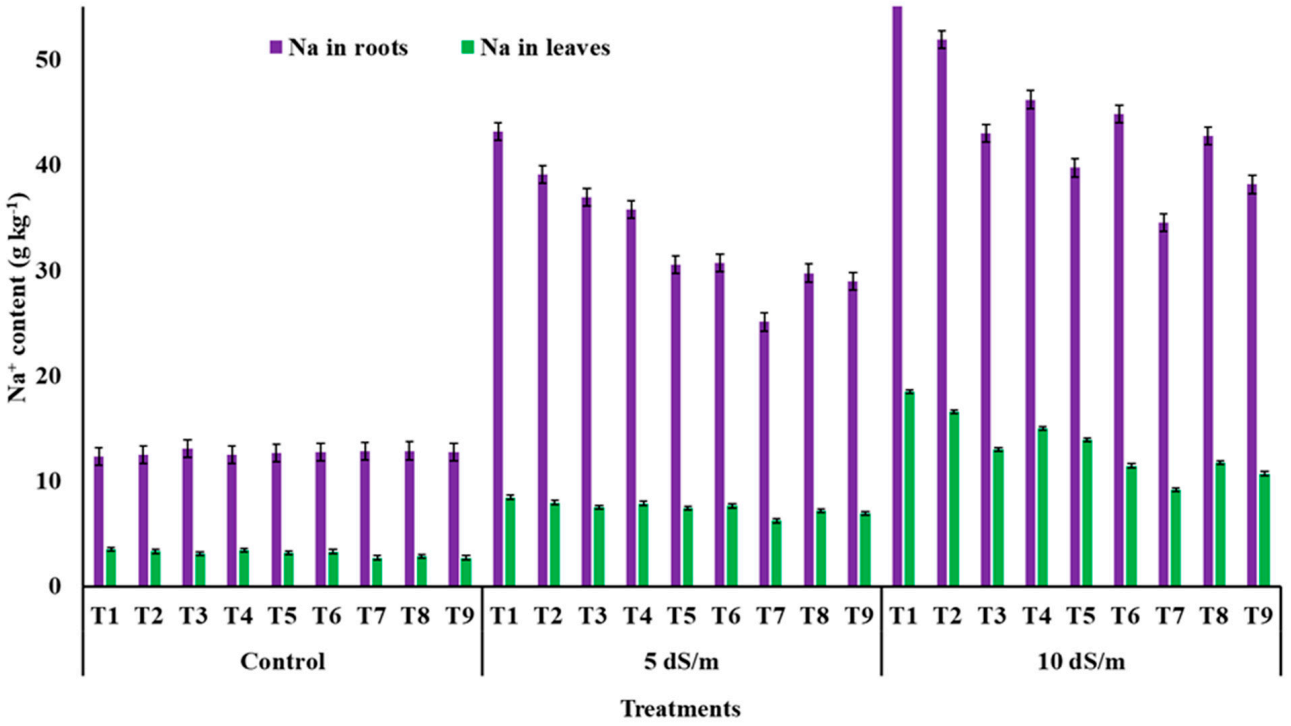

Figure 7. Effect salinity and root application of EBL and ZnO-NPs on the Na^+ content in both roots and leaves of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

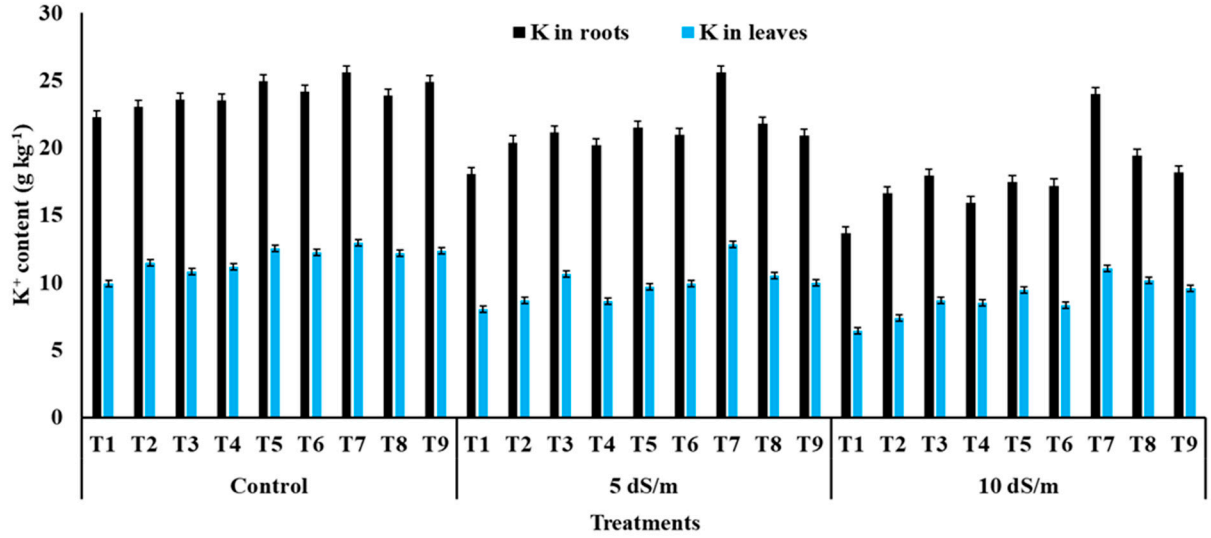

Figure 8. Effect salinity and root application of EBL and ZnO-NPs on the K^+ content in both roots and leaves of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

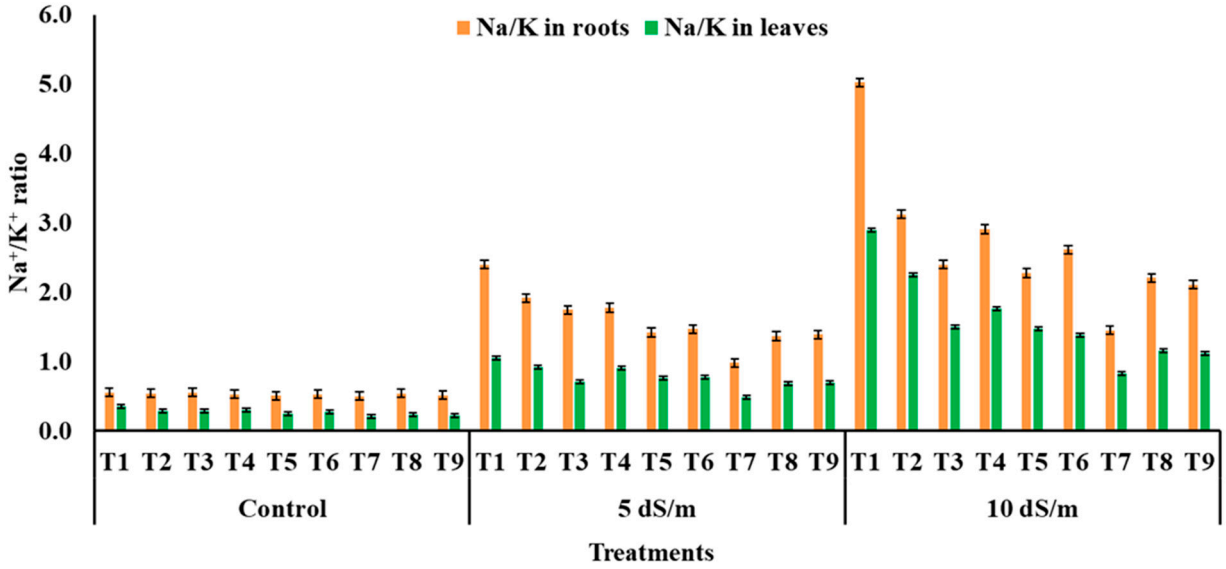

Figure 9. Effect salinity and root application of EBL and ZnO-NPs on the Na^+/K^+ ratio content in both roots and leaves of maize plants. Error bars represents square error mean (SEM at 0.05). See Figure 1 for abbreviations.

The results showed a highly significant interaction between EBL and ZnO-NPs application treatments and saline stress. The lowest Zn^{2+} content in roots (40.05 g kg^{-1} DM) and leaves (33.15 g kg^{-1} DM) was recorded for T3 and T2, respectively, under high (10 dS m^{-1}) saline stress, whereas the maximum was found in roots (154.47 g kg^{-1} DM) and leaves (132.59 g kg^{-1} DM) for T9 and T5, respectively, in unstressed plants (control). Root application of T7 and T9 improved Zn^{2+} absorption even under high (10 dS m^{-1}) saline stress, i.e., +50% and +52% in roots, and +15% and +15% in leaves, respectively, in comparison with the control (T1 + 0 dS m^{-1}) (Figure 6). Treatment control (T1) under high (10 dS m^{-1}) saline stress resulted in the lowest K^+ content for both roots (13.65 g kg^{-1} DM) and leaves (6.40 g kg^{-1} DM), whereas T7 elevated it by +200% and +153% in roots and leaves, respectively (Figure 8). T1 under high (10 dS m^{-1}) saline stress resulted in maximum Na^+ content in both samples (roots and leaves) as 68.46 and 18.46 g kg^{-1} DM, respectively.

However, T8 and T9 managed to decrease them by −38% and −44% in roots, and −36% and −42% in leaves, respectively (Figure 7). Interestingly, T7 under saline control (0 dS m^{-1}) significantly minimized the Na^+/K^+ ratio to its lowest in roots (0.50) and leaves (0.21), whereas the maximum was noted for T1 under 10 dS m^{-1} as 5.02 and 2.89 for roots and leaves, respectively (Figure 9).

The ionic uptake (i.e., Na^+, K^+ and Zn^{2+}) was particularly focused, and its concentration in both roots and leaves was measured. Under saline stress conditions, the Na^+ mound in roots ultimately reaches the plant's upper parts via the transpiration stream, where it amasses in various compartments of the leaf cells [84]. At transport sites, Na^+, having a nearly similar hydrated radius as that of K^+, competes with other Na^+ and lowers its uptake by the root cells. The succeeding lower K^+ concentration in the plant cells widens the Na^+/K^+ ratio and ultimately results in a physiological imbalance in a number of ways [85]. In saline stress *Arabidopsis*, Na^+ toxicity down-regulated the genes related to K^+ inward-rectification and hence disrupted the Na^+/K^+ balance [86]. At a cellular level, K^+ is essential to maintain cell turgidity, enzymatic activity, and metabolism, thus K+ deficiency limits the plant growth [87]. The mechanism of Na^+ stress on Zn^{2+} ion uptake, however, is not fully understood yet.

Zinc application in the form of ZnO-NPs facilitates saline stress tolerance in plants by reducing the Na^+ absorption, and by promoting K^+ active absorption [88,89]. El-Badri et al. [90] reported, ZnO-NPs reduced Na^+ accumulation in cell cytosol and enhanced K^+, Zn^{2+}, and Ca^{2+} concentration in root cells. Foliar application of ZnO-NPs at 100 mgL^{-1} improved nutrient uptake both for macronutrients (i.e., K^+, Ca^{2+}, P, and Mg^{2+}) and micronutrients (i.e., Zn^{2+} and Fe^{2+}) in *Solanum tuberosum* L. under drought stress [18]. Brassinosteroids as plant growth regulators controlled the inorganic ions uptake by decreasing toxic accumulation of excess ions in roots and leaves in *Brassica napus* L. under saline stress [31]. Furthermore, exogenous application of BR enhanced the enzymatic activity of H^+-ATPase and Ca^{2+}-ATPase responsible for maintenance of electrochemical gradient and ionic balance to overcome various abiotic stresses in plants. Under 200 mM saline stress, EBL application significantly reduced Na^+ content and enhanced K^+ and Ca^{2+} accumulation in *Robinia pseudoacacia* L. [72]. Kapoor et al. [91] reported EBL as a mineral ion regulator under different heavy metal stresses, such as Cd and Hg.

4. Conclusions

Salt stress negatively affected growth and the physiological and biochemical traits of maize, as well as impacted the elemental uptake. However, root application of EBL and ZnO-NPs, either as a single or in combination, mitigated the negative effects of saline stress and enhanced plant growth. The combination treatment of 0.02 µM EBL + 100 mg/L ZnO-NPs resulted in the highest improvements in terms of growth and the physiological and biochemical traits of maize due to their role in mitigating salt stress. For instance, the combination of 0.02 µM EBL + 100 mg/L ZnO-NPs increased root length by +175%, shoot length by +39%, leaf area by +181%, RWC by +12%, net photosynthesis by +275, total chlorophyll content by +33%, and total phenolic content by +38% in comparison to those obtained from the control, respectively. In conclusion, the root application of ZnO-NPs (i.e., 100 mg/L) in combination with EBL (i.e., 0.02 µM) can be used to mitigate salt stress in hydroponic systems and improve the performance of maize plants.

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