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Effect of Plant Growth Stimulants on Alfalfa Response to Salt Stress

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Abstract

Salinity is a major impediment to crop production. This study was undertaken to compare the effect of seaweed extract, humic acid, and potassium sulfate nanoparticles in alleviating salt stress in Alfalfa (*Medicago sativa* L.). Seeds of ten alfalfa genotypes were germinated in a growth chamber at five salt concentrations (0%, 0.5%, 1.0%, 1.5%, and 2.00%). Salt concentrations above 1% reduced seed germination by more than 70% in most genotypes. One salt tolerant genotype (Mesa-Sirsa) and one salt sensitive (Bulldog 505) were selected and planted in greenhouse pots containing 2 kg of sand and subjected to two salt levels (10 and 15 $dS \cdot m^{-1}$). Four treatments consisting of 1) control (Hoagland solution, no-salt), 2) seaweed extract at 4 $Kg \cdot ha^{-1}$, 3) humic acid at 28 $L \cdot ha^{-1}$, and 4) potassium sulfate at 300 $Kg \cdot ha^{-1}$. Plant biomass was reduced under both salt concentrations in both genotypes, with a greater magnitude in the salt sensitive genotype. Application of seaweed extract resulted in higher relative water content and proline under both salt concentrations (10 and 15 $dS \cdot m^{-1}$) in the salt sensitive genotype, and lower electrolyte leakage in both salt tolerant and salt sensitive genotypes, under both salt concentrations. Seaweed extract also resulted in higher catalase and SOD activities in both genotypes under 10 $dS \cdot m^{-1}$. Catalase and SOD activities were associated with significantly ($p < 0.01$) reduced electrolyte leakage and increased shoot dry weight. Overall, seaweed extract seemed to have a positive effect in alleviating salt stress in alfalfa.

Keywords

Biological Growth Stimulants, Humic Acid, Salt Stress, Seaweed Extract, Potassium Nanoparticles

1. Introduction

About 900,000 ha of Egypt's agricultural lands are suffering from salinity build-

up problem [1]. The majority of salt-affected areas are located in the northern-central part of the Nile Delta and on its eastern and western sides. Sixty percent of the cultivated land of the Northern Delta region, 20% of the Southern Delta and Middle Egyptian region, and 25% of the Upper Egypt regions are salt-affected [2]. Salinity is one of the most limiting factors to crop production in arid and semiarid regions of the world. Globally, it affects an estimated 6% of the world's land area or 12,780 million hectares (Mha), in addition to an estimated 20% of irrigated land impacted by secondary salinization resulting from irrigation [3] [4]. Salinity reduces plant growth and production by affecting physiological processes, including disruption of ionic equilibrium, water status, mineral nutrition, stomatal behavior, and photosynthetic efficiency [5]. Disparity in Osmotic potential leads to water deficit, reduced leaf area expansion and stomatal closure, which ultimately reduces photosynthesis and plant growth [6]. The ionic disequilibrium causes excessive accumulation of Na^+ and Cl^- in the older leaves, leading to their premature senescence [6] [7], in addition to creating an ionic imbalance that reduces the uptake of beneficial ions such as K^+, Ca^{2+}, and Mn^{2+} [8] and inhibits photosynthesis and enzyme activities [9].

Several studies carried out to elucidate salinity tolerance mechanisms included the application of biological stimulants and organic acids [10]. Seaweed extract is considered a source of organic matter and nutrients and is used as a soil conditioner [11] [12]. Seaweed extracts have been used in agriculture and horticulture as bio-stimulants to promote plant growth and increase crop yields [13]. Zhang and Ervin [14] provided evidence that plant performance was improved under water stress conditions upon treatment with seaweed extracts. Seaweeds have been reported to possess plant-growth promoting activity which made them relevant in agriculture and horticulture as organic fertilizers [15]. Fike *et al*. [16] reported that seaweed extract derived from A. *nodosum* contains various compounds including amino acids and micronutrients. The application of Seaweed extract on vegetables and forage grasses increased root length, leaf area, and root and shoot biomass in response to drought stress, in addition to increasing chlorophyll and carotenoids [17]. In general, seaweed extracts are capable of inducing an array of physiological plant responses, such as the promotion of plant growth, improvement of flowering and yield, and enhancing nutritional quality of edible products as well as shelf life, even at low concentrations. Furthermore, applications of different extract types have been reported to enhance plants' tolerance to a wide range of abiotic stresses, such as salinity, drought, and temperature extremes [12]. The effect of seaweed extracts in alleviating salt stress in Alfalfa has not been investigated. Having been derived from a renewable resource, the bioactive extracts from seaweed could be useful on a large scale to improve alfalfa productivity and sustainability of agricultural systems if proven effective in overcoming salt stress.

Humic substances have shown anti-stress effects under abiotic stress conditions such as unfavorable temperature, salinity, and low pH [18]. Humic acid promotes plant growth by enhancing the uptake of nutrients and reducing the

uptake and accumulation of some toxic elements. Increasing cell membrane permeability, oxygen uptake, photosynthesis, phosphate uptake, and root cell elongation are some of the factors suggested to explain the positive effect of humic acid on plant growth [19]. Humic acid has the ability to chelate various nutrients including Na, K, Mg, Zn, Ca, Fe, and Cu in order to overcome nutrient deficiencies [20] [21].

Plants require K for a number of important physiological processes including the activation of various enzymes, the synthesis and metabolism of carbohydrates, protein synthesis, and the opening and closing of stomata thus controlling gas exchange and photosynthesis [22]. The application of K^+ has been shown to mitigate the adverse effects of salinity through its roles in stomatal regulation, osmotic adjustment, and maintenance of the membrane ion-charge balance, cellular-energy status, and protein synthesis [23].

Alfalfa (*Medicago sativa* L.), is an important forage crop grown over 32 million hectares globally [24]. It is a high yielding perennial forage grown in different climates all over the world [25]. Alfalfa is highly sought after for its high crude protein content and total digestible nutrients [26] [27]. Alfalfa has been characterized as moderately sensitive to salts with an electrical conductivity (EC) of 2.0 $dS \cdot m^{-1}$ (1280 ppm) and a threshold of 1.5 bars (1 bar = 0.987 atmospheres) osmotic potential of soil solution at field capacity [28].

The primary objective of this study was to explore the effect of different growth-enhancing substances, such as seaweed extract, humic acid, and potassium sulfate, on alfalfa growth and physiological response under salt stress.

2. Materials and Methods

2.1. Alfalfa Seed Germination under Salt

Alfalfa seeds were germinated in 100-mm petri plates containing a single piece of Whatman No. 2 filter paper imbibed with two types of saline solutions. The first solution consisted of NaCl and the second was a mixture of (NaCl, $MgCl_2 \cdot 6H_2O$ and Na_2SO_4), the most common active ingredients in seawater. Each saline solution was added in five concentrations 0%, 0.5%, 1%, 1.5% and 2% (wt/wt) in deionized water. Twenty-five scarified seeds from each of ten alfalfa genotypes that were described as salt tolerant (Malone, Mesa-Sirsa, Rambler, and Saranac) and salt sensitive (Barricade, Bulldog 505, Hybriforce 2600, Magna 801FQ, 3010, and CW1010) were placed in each petri plate. Four and a half (4.5) mL of each salt solution with the appropriate concentration was added to each plate. The experimental design was a split-plot in a randomized complete block design with three treatments. Salt solutions represented the main plots and the increasing concentrations represented the sub-plots. The germination test was conducted in a dark growth chamber at 25˚C for 14 days. Seed germination percentage (*GP*) was calculated as:

$$GP = 100(G/TS) \quad (1)$$

where G = number of germinated seeds, and TS = total number of seeds.

2.2. Whole-Plant Response to Salt Stress

One alfalfa salt-tolerant genotype (Mesa-Sirsa) and one salt-sensitive (Bulldog 505) were selected based on the results of the seed germination experiment described above. Six seeds from each genotype were planted in pots containing 2 kg sand and lined with plastic bags. Seedlings within each pot were thinned after four weeks from planting to keep four plants per pot. The plants were gradually subjected to two levels of salt concentrations starting at five weeks after planting. Calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium chloride (NaCl) were mixed in a 2:1 proportion ($CaCl_2$:NaCl) and added to Hoagland solution to make two saline nutrient solutions of 10 and 15 $dS \cdot m^{-1}$ electrical conductivity. The moisture level in the pots was kept at field capacity. Four treatments consisting of 1) control (Hoagland solution), 2) seaweed extract from *Ascophyllum nodosum* (Organic Approach, Lancaster, PA) at 4 $Kg \cdot ha^{-1}$, (3) humic acid (KELP4LESS, Idaho Falls, ID) at 28 $L \cdot ha^{-1}$, and 4) potassium sulfate nanoparticles at 300 $kg \cdot ha^{-1}$. Humic acid and seaweed extract were applied once at one week after the start of salt treatments, whereas potassium sulfate was added in 2 split applications, the first application was one week following salt application and the second at bud stage. All treatments were irrigated with Hoagland solution when needed. Plants were harvested 90 days after planting. Soil characteristics and chemical composition were recorded at the beginning of the study (Table 1) and at the end of the study. Plant biomass was measured as shoot and root dry weights using a digital scale with 0.001 g sensitivity. Root length in cm, number of tillers, and relative yield were also recorded.

Relative water content (*RWC*) of shoots was measured according to Turner [29] using the equation:

$$RWC(\%) = (FW - DW)/(TW - DW) \quad (2)$$

where, FW = fresh weight, TW = turgor weight, and DW = dry weight. Dry weight was estimated by drying the samples in a convection oven at 80˚C for 48 h. Turgor weight was determined by floating the shoots on water at room

Table 1. Physical characteristics and chemical composition of the soil used in the evaluation of two Alfalfa genotypes grown under three salt levels (0, 10, and 15 $dS \cdot m^{-1}$ EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Sand	**100.0%**	**Mineral concentration ($mg \cdot kg^{-1}$)**								
Silt	0.0%	Ca	Mg	Na	K	Cl	NH_4-N	NO_3-N	P	Fe
Clay	0.0%	35.17	5.94	<1.79	2.55	8.70	<6.40	<0.17	0.97	5.46
Soil Type	Sand									
EC[1]	0.011 $dS \cdot m^{-1}$	Zn	B	Mn	Cu	Mo	Cd	Cr	Ni	Pb
pH	6.68									
CEC[2]	0.21 meq. 100 g^{-1}	0.33	0.775	0.88	0.29	<0.04	<0.04	0.04	<0.04	0.36
OM[3]	0.01%									

[1]EC: Electrical conductivity in $dS \cdot m^{-1}$. [2]CEC: Cation exchange capacity in meq.100 g^{-1} soil. [3]OM: Organic matter.

temperature for 48 h. Relative yield was determined according to Isla & Aragüés [30] by dividing the actual yield in each saline treatment by the highest yield observed.

2.2.1. Physiological Response to Salt Stress

Free proline content in plant tissue was determined according to the methods of Bates *et al*. [31], where 100 mg of plant tissue were homogenized in 2 ml of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 13,000 ×g for 10 mn. Then, 1 ml of supernatant was placed in a test tube and reacted with 1 ml of acid ninhydrin and 1 ml glacial acetic acid. The test tubes were heated in boiling water in a warm bath for 1 h, and the reaction was terminated by placing the test tubes in an ice bath. The reaction mixture was extracted with 2 ml toluene and mixed vigorously by vortexing. The toluene layer was separated at room temperature and the absorbance of chromophore containing toluene was measured at 520 nm on a Varian Cary 50 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA), using pure toluene as the blank. Standard curves were prepared for each assay using standard proline in 3% sulfosalicylic acid solution. The proline content was expressed as micromoles per gram of fresh weight plant material (μmol·g^{-1} FW).

Electrolyte leakage (*EL*) was determined as described by Lutts *et al*. [32]. Fresh leaves (200 mg) from the alfalfa plants were placed in test tubes containing 10 ml of deionized water. The tubes were incubated at 25˚C on a rotary shaker for 24 hours and, subsequently, the electrical conductivity of the solution (L_t) was measured. The samples were then autoclaved at 120˚C for 20 mn, and the final electrical conductivity (L_0) was determined after equilibration at 25˚C. Measurements of electrical conductivity were made using H1993310 conducti-meter (HANA Instruments, Romania). Electrolyte leakage was expressed as:

$$EL(\%) = (L_t / L_0) \times 100 \quad (3)$$

2.2.2. Antioxidant Enzymes Analysis

Tissue samples of 0.2 g from each leaf were homogenized with 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 2% (w/v) polyvinylpyrrolidone (PVP). The entire extraction procedure was carried out at 4˚C. The homogenate was centrifuged at 10,000 g for 15 mn at 4˚C and the supernatant was collected and used for assaying enzyme activity.

Catalase (CAT, EC 1.11.1.6) activity was measured according to Bergmeyer & Gawehn (1970) [33] as the rate of H_2O_2 disappearance at 240 nm by adding 100 μl leaf crude extract to the solution mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 2% H_2O_2. Enzyme activity was calculated as units of H_2O_2 consumed per minute and per gram fresh weight (mmol H_2O_2 min^{-1}·g^{-1} FW).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed spectrophotometrically as the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm according to the method of Beauchamp & Fridovich (1971)

[34]. The reaction mixture of 3 ml volume, consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 10 μM EDTA, 2.0 μM riboflavin and 0.3 ml enzyme extract. The test tubes containing reaction mixtures were weighed for 10 mn under 4000 lx at 35˚C. One-unit SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the rate of NBT reduction measured at 560 nm.

2.3. Statistical Analysis

Analysis of variance (ANOVA) was conducted on the data from the two experiments using PROC GLM of SAS 9.4 (SAS Institute Inc., Cary, NC). Replications were considered random and all other variables were considered fixed effects. Means of all variables were separated using Fisher's protected LSD test.

3. Results and Discussion

3.1. Alfalfa Seed Germination under Increasing Salt Concentrations

There were significant differences between genotypes in their response to the type of salt solution and to the increase in salt concentration. Increasing salt concentrations significantly affected ($p < 0.01$) seed germination after two weeks in the 10 alfalfa genotypes (Table 2). The germination percentage under NaCl salt solution decreased at a faster pace in all genotypes with increasing salt concentration compared to the mixed salts solution (Table 3). At the lower salt levels, the reduction in germination varied from 0% to 78.05% at the 0.5% NaCl concentration and from 58.69% to 95.52% at the 1.0% NaCl level compared to no-salt control. Increasing NaCl concentration to 1.5 % resulted in a decrease of germination ranging from 70.83% to 100% compared to no-salt control. At 2% salt level, the magnitude of reduction in germination varied from 93.75% to 100% compared to the no-salt control. Under mixed salts solutions, the response in germination was different from NaCl solution, as 0.5% and 1.0% mixed salt concentrations enhanced the germination percentage by 14.03% and 9.52% more

Table 2. Mean squares and significance of seed germination of ten alfalfa genotypes after two weeks, in response to two salt solutions of NaCl and mixed salts (NaCl, $MgCl_2 \cdot 6H_2O$ and Na_2SO_4) at five different concentrations (0%, 0.5%, 1%, 1.5%, and 2%) each.

Source of variation	Degrees of Freedom	Mean Squares
Genotypes	9	1206.58**
Salt solution	1	33,606.67**
Salt conc.	3	75.87**
Genotype Salt source	9	70,772.34
Genotype & Salt Conc.	27	298.00**
Salt source & Salt conc.	3	9109.41**
Genotype & Salt source & Salt conc.	27	338.81**
Error	180	

*, ** Significant at 0.05 and 0.01 probability level.

Table 3. Percent seed germination of ten alfalfa genotypes after two weeks, in response to two salt solutions (NaCl and mixed NaCl, $MgCl_2 \cdot 6H_2O$ and Na_2SO_4) at five different concentrations (0%, 0.5%, 1%, 1.5%, and 2%) each.

Genotypes	NaCl (%)					Mixed (NaCl, $MgCl_2 \cdot 6H_2O$ and Na_2SO_4) (%)				
	0	0.5	1.0	1.5	2.0	0	0.5	1.0	1.5	2.0
3010	93.3 ± 2.3	66.7 ± 2.3	14.0 ± 2.0	3.0 ± 1.7	6.0 ± 2.0	93.3 ± 2.3	96.0 ± 0.0	92.0 ± 4.0	20.0 ± 4.0	0.0 ± 0.0
Bulldog505	82.0 ± 2.0	18.0 ± 2.0	4.0 ± 0.00	0.0 ± 0.0	0.0 ± 0.00	82.0 ± 2.0	74.0 ± 2.0	67.7 ± 0.6	3.7 ± 0.6	0.0 ± 0.0
Barricade	92.0 ± 0.0	84.0 ± 4.0	22.7 ± 2.3	11.0 ± 1.0	0.0 ± 0.00	92.0 ± 0.0	93.3 ± 2.3	88.0 ± 4.0	16.7 ± 4.2	0.0 ± 0.0
CW1010	92.0 ± 6.9	82.7 ± 4.6	38.0 ± 2.0	7.7 ± 0.6	0.0 ± 0.0	92.0 ± 6.9	88.0 ± 4.0	85.3 ± 2.3	34.0 ± 3.5	0.0 ± 0.0
Hybriforce2600	90.0 ± 2.0	58.0 ± 2.0	16.3 ± 4.0	7.0 ± 1.0	3.7 ± 0.56	90.0 ± 2.0	86.7 ± 4.6	84.0 ± 4.0	18.0 ± 2.0	0.0 ± 0.0
Magna801FQ	84.0 ± 0.0	84.0 ± 4.0	34.02.00	27.3 ± 8.1	0.0 ± 0.0	84.0 ± 0.0	84.7 ± 1.2	92.0 ± 8.0	25.3 ± 2.3	0.0 ± 0.0
Malone	70.0 ± 10.0	66.0 ± 6.0	27.3 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	70.0 ± 10.0	79.7 ± 0.6	73.3 ± 4.6	30.0 ± 2.0	0.0 ± 0.0
Mesa-Sirsa	96.0 ± 0.0	86.0 ± 2.0	38.0 ± 2.0	28.0 ± 2.0	6.0 ± 0.0	96.0 ± 0.0	98.7 ± 2.3	93.3 ± 4.6	45.3 ± 3.1	6.0 ± 2.0
Rambler	89.3 ± 2.3	50.7 ± 9.2	4.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	89.3 ± 2.3	78.0 ± 2.0	38.7 ± 10.1	4.3 ± 0.6	0.0 ± 0.0
Sarance	76.0 ± 0.0	66.0 ± 2.0	16.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0	76.0 ± 0.0	86.7 ± 6.1	78.0 ± 2.0	24.0 ± 4.0	3.3 ± 0.6

than the no-salt control (Table 3). At 1.5% mix salts concentration, the reduction in seed germination percentage varied from 52.78% to 95.52%, while at 2% concentration the decrease ranged from 93.75% to 100% compared to the no-salt control. This trend was observed in other crops exposed to salt stress [35] [36] [37]. The genotype Mesa-Sirsa exhibited the highest tolerance to increasing salt concentrations, whereas Bulldog 505 genotype was the most susceptible of the 10 genotypes tested. Germination percentage of Mesa-Sirsa decreased from 96% under no-salt to 28% and 6% at 1.5% and 2% NaCl solution, and to 45.33% and 6% at 1.5% and 2% mixed salt solution after 2 weeks. Whereas Bulldog 505, grown under 1.5% and 2% NaCl or mixed salts showed no germination after 2 weeks (Table 3).

3.2. Greenhouse Experiment

3.2.1. Soil Properties

An overall change in soil properties was observed at the end of the study, relative to the no-salt control (Table 4). There was a significant difference ($p < 0.01$) among genotypes in pH, Ca, K, Mg, B, Zn, and Fe. Salt concentrations had a significant effect ($p < 0.01$) on pH, EC, CEC, Ca, K, Mg, Na, B, and Zn. The application of growth enhancing treatments resulted in significant changes ($p < 0.01$) in EC, Ca, K, Mg, Zn, Fe, and Mn (Table 4). The application of humic acid reduced the pH under both salt levels (10 and15 $dS \cdot m^{-1}$), with Bulldog recording 6.7 and 6.63 respectively, compared to the no-salt control, while seaweed extract resulted in the lowest pH with the tolerant genotype Mesa-Sirsa under both salt levels (6.57 and 6.48, respectively). The EC increased by 3 to 4 folds with increasing salinity levels under the 10 $dS \cdot m^{-1}$ salt level and by 4 to 6 folds under the 15 $dS \cdot m^{-1}$ salt level, regardless of the growth enhancing treatment applied (Table 5). Aydin *et al.* (2012) [19] reported increases in EC of the soil with increasing salt concentrations with the highest increase was at 120 mM NaCl concentration. Potassium sulfate enhanced CEC of the soil under both alfalfa geno-

Table 4. Mean squares for various soil properties and mineral concentrations (mg·kg^{-1}) measured after the evaluation of two Alfalfa genotypes grown under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Source	DF[2]	pH	EC[3]	CEC[4]	Ca	K	Mg	P	Na	B	Zn	Fe	Mn
Genotypes	1	0.2**	0.06	0.1	9960.4*	197.9*	379.9**	1.2	453.7	0.1**	0.1**	4.1**	0.01
Salt Conc.	1	0.2**	4.22**	24.8**	470,472.5**	1141.0**	256.2**	2.2	181,838.3**	0.1**	0.2**	0.01	0.0
Treatments[1]	2	0.01	0.54**	0.2	8081.2**	3375.5**	28.5**	1.6	485.9	0.01	0.2**	0.2*	0.2**
Genotypes × Salt Conc.	1	0.1**	0.12	14.5**	311,639.3**	12,019.8**	928.2**	525.7**	22,910.4**	0.2**	0.2**	3.6**	0.6**
Genotypes × Treat	2	0.001	0.05	1.8**	36,539.3**	253.4**	40.2**	78.8**	1765.3*	0.01	0.2	0.8**	0.1**
Salt Conc. × Treat	2	0.01	1.24**	13.0**	263,097.3**	8606.7**	324.0**	259.7**	18,742.4**	0.1**	0.2	8.1**	1.1**
Genotypes × Salt Conc. × Treat	2	0.04**	0.46**	7.8**	125,630.1**	6000.1**	179.6**	117.0**	16,894.4**	0.1**	0.2	1.9**	0.1**
Error		0.002	0.036	0.1	1043.7	44.7	3.4	2.4	363.9	0.01	0.01	0.1	0.0
LSD[5]		0.03	0.120	0.2	20.4	4.2	1.2	1.0	12.1	0.1	0.1	0.2	0.1

*, **Significant at 0.05 and 0.01 probability level. [1]Treatments: growth-enhancing substances (seaweed extract, humic acid, potassium sulfate). [2]DF: Degrees of freedom. [3]EC: Electrical conductivity in dS·m^{-1}. [4]CEC: Cation exchange capacity in meq 100 g^{-1} soil. [5]LSD: Least significant difference at $\alpha = 0.05$.

types at 10 dS·m^{-1} recording 69.4% with Bulldog, and 80.4% with Mesa-Sirsa, compared to no-salt control. Under 15 dS·m^{-1}, seaweed extract increased the CEC of the soil under the susceptible genotype (Bulldog) recording 9.2 meq 100 g^{-1}, while humic acid treatment recorded the highest CEC with the tolerant genotype (Mesa-Sirsa) with a value of 7.5 meq 100 g^{-1}. The application of potassium sulfate resulted in accumulations of Ca, Mg, K, P, Na, B, Zn, Fe and Mn in the soil under both alfalfa genotypes at10 dS·m^{-1}, while under 15 dS·m^{-1}, seaweed extract resulted in accumulation of Ca, Mg, K, P, Na, B, Zn, Fe and Mn in the soil under both alfalfa genotypes.

3.2.2. Plant Tissue Chemical Characteristics

Salt concentrations significantly affected ($p < 0.01$) the mineral composition of plant tissue in both genotypes and increased the Na/K ratio compared to the no-salt control (**Table 6**). Increasing the salt concentration to 10 dS·m^{-1} resulted in an increase in the Na/K by a 32 fold in the susceptible genotype and 36 fold in the tolerant genotype, compared to the no-salt control (**Table 7**). A similar trend was reported in rice grown under salt stress [38]. Na/K ratio is an important consideration in salt tolerance and salt-stress alleviation because reducing Na^+ uptake and transport from roots to shoots and increasing retention of K^+ in the cytosol are considered key factors in conferring salt tolerance in plants [39] [40].

There was a significant difference ($p < 0.01$) among the genotypes in Na/K and the concentration of Ca, Mg, N, S, Mn, Cu and Al in plant tissue. In the sensitive genotype, the application of potassium sulfate under salt stress at 10 dS·m^{-1} enhanced the uptake of K ions rather than Na^+ ions leading to a lower Na/K compared to the other treatments. Humic acid treatment reduced the Na/K ratio under the higher salt concentration (15 dS·m^{-1}). In the salt tolerant genotype, the Na/K ratio was lower than other treatments under the 10 dS·m^{-1} salt level,

Table 5. Soil characteristics after harvesting the plants of two Alfalfa genotypes evaluated under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Genotypes	Salt level	Treatments	pH[2] $CaCl_2$	EC[1]	CEC[2]	Ca	K	Mg	P	Na	B	Zn	Fe	Mn
				dS·m^{-1}	Meq. 100 g^{-1}	mg·kg^{-1}								
Bulldog 505	0 dS·m^{-1}	Control (0 salt)	6.9 ± 0.07	0.6 ± 0.02	1.9 ± 0.05	181.7 ± 8.5	150.1 ± 3.9	71.1 ± 3.2	23.5 ± 1.5	8.93 ± 0.8	0.47 ± 0.1	0.18 ± 0.006	3.9 ± 0.4	1.8 ± 0.3
	10 dS·m^{-1}	Seaweed extract	6.9 ± 0.01	2.1 ± 0.04	3.5 ± 0.04	492.4 ± 5.5	103.6 ± 3.7	24.5 ± 0.3	15.7 ± 0.7	154.4 ± 3.6	0.25 ± 0.03	0.19 ± 0.0	2.4 ± 0.0	0.7 ± 0.08
		Humic acid	6.7 ± 0.06	2.5 ± 0.29	3.8 ± 0.03	511.9 ± 9.0	108.3 ± 3.2	25.7 ± 0.6	16.0 ± 3.3	156.2 ± 0.2	0.24 ± 0.3	0.19 ± 0.0	1.8 ± 0.16	0.9 ± 0.1
		Potassium sulfate	6.8 ± 0.03	1.8 ± 0.02	6.2 ± 0.04	819.6 ± 4.5	213.3 ± 2.2	44.1 ± 0.6	27.7 ± 1.4	288.6 ± 6.4	0.39 ± 0.06	0.19 ± 0.006	4.4 ± 0.04	1.4 ± −0.1
	15 dS·m^{-1}	Seaweed extract	6.5 ± 0.0	3.0 ± 0.04	9.2 ± 0.20	1279.6 ± 12.6	195.4 ± 0.3	42.0 ± 0.69	36.9 ± 0.02	457.2 ± 0.3	0.53 ± 0.01	0.19 ± 0.0	4.2 ± 0.3	1.5 ± 0.2
		Humic acid	6.6 ± 0.03	2.6 ± 0.20	8.03 ± 0.06	1100.6 ± 2.5	178.5 ± 3.0	37.5 ± 0.5	25.2 ± 0.06	411.4 ± 8.8	0.43 ± 0.04	0.20 ± 0.006	3.9 ± 0.04	1.3 ± 0.2
		Potassium sulfate	6.6 ± 0.07	2.6 ± 0.15	5.1 ± 0.63	688.0 ± 80.0	127.1 ± 16.8	29.2 ± 3.6	18.6 ± 1.99	308.4 ± 67.4	0.36 ± 0.09	0.2 ± 0.0	2.3 ± 0.3	0.9 ± 0.3
Mesa Sirsa	0 dS·m^{-1}	Control (0 salt)	6.7 ± 0.06	0.6 ± 0.03	1.3 ± 0.07	139.0 ± 9.0	110.3 ± 5.2	36.8 ± 1.3	33.2 ± 1.3	6.7 ± 0.8	0.38 ± 0.04	0.2 ± 0.06	4.5 ± 0.2	1.5 ± 0.02
	10 dS·m^{-1}	Seaweed extract	6.6 ± 0.05	1.8 ± 0.09	5.8 ± 0.29	813.2 ± 16.7	174.2 ± 3.6	43.6 ± 2.9	21.6 ± 0.6	253.4 ± 8.5	0.36 ± 0.07	0.2 ± 0.006	3.9 ± 0.2	1.2 ± 0.08
		Humic acid	6.6 ± 0.03	2.5 ± 0.04	5.9 ± 0.23	816.4 ± 14.4	167.8 ± 4.7	43.2 ± 2.0	24.8 ± 1.7	249.8 ± 6.1	0.34 ± 0.05	0.2 ± 0.0	3.9 ± 0.5	1.4 ± 0.1
		Potassium sulfate	6.6 ± 0.02	2.1 ± 0.006	6.63 ± 0.27	924.1 ± 21.8	214.5 ± 1.7	47.1 ± 1.9	27.4 ± 2.2	277.2 ± 3.4	0.37 ± 0.05	0.2 ± 0.006	4.7 ± 0.04	1.6 ± 0.1
	15 dS·m^{-1}	Seaweed extract	6.5 ± 0.11	3.7 ± 0.37	5.75 ± 0.67	818.3 ± 53.5	118.4 ± 7.6	25.4 ± 1.9	14.5 ± 1.5	322.8 ± 6.8	0.28 ± 0.06	0.22 ± 0.03	3.04 ± 0.2	1.1 ± 0.06
		Humic Acid	6.6 ± 0.02	2.6 ± 0.26	7.51 ± 0.37	1033.9 ± 62.1	140.2 ± 7.7	34.5 ± 1.3	20.2 ± 1.2	373.4 ± 11.9	0.31 ± 0.07	0.2 ± 0.0	4.3 ± 0.3	1.6 ± 0.2
		Potassium sulfate	6.6 ± 0.01	2.5 ± 0.02	6.25 ± 0.03	829.1 ± 8.8	154.4 ± 10.5	27.5 ± 0.7	14.7 ± 0.7	359.1 ± 10.4	0.31 ± 0.01	1.06 ± 0.3	3.1 ± 0.2	0.7 ± 0.02

Mineral elements concentration in mg·kg^{-1}. [1]EC: Electrical conductivity in dS·m^{-1}. [2]CEC: Cation exchange capacity in meq 100 g^{-1} soil.

Table 6. Mean squares for chemical composition of the plant tissue of two Alfalfa genotypes evaluated under three salt levels (0, 10, and 15 dS·m^{-1} electrical conductivity) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Source	DF[1]	Na/K	Ca	Mg	P	N	S	Fe	Zn	Mn	Cu	B	Al
Genotypes	1	73.7**	8.1**	0.1**	0.001	1.8**	0.04**	100.9	2.8	316.5**	0.75**	15.1	90.04**
Salt Conc.	1	787.0**	4.5**	0.1**	0.09**	3.2**	0.03**	1840.4**	193.3**	63.5**	6.7**	90.57**	1931.60**
Treatments[2]	2	2.6	4.0**	0.01	0.005	0.01	0.007*	736.2**	98.3**	36.8**	1.7**	39.0**	422.58**
Genotypes × Salt Conc.	1	38.8*	6.8**	0.00002	0.001	1.9**	0.04	20.3	0.79	36.4**	0.01	20.1*	67.51**
Genotypes × Treatments	2	43.2**	0.3	0.001	0.01**	0.09	0.02**	54.7	3.0	64.5**	1.6**	40.5**	340.39**
Salt Conc. × Treatments	2	23.9*	0.5**	0.003	0.00003	0.01	0.008*	329.6**	2.4	46.3**	0.78**	5.1	452.07**
Germpl × Salt Conc. × Treatments	2	23.4*	1.4	0.005	0.009*	0.3**	0.0003	434.01**	44.7	70.3**	3.04**	42.1**	383.63**
Error		6.23	0.5	0.004	0.002	0.05	0.002	31.32	2.49	5.24	0.09	4.70	3.39
LSD[3]		1.58	1.4	0.12	0.09	0.4	0.086	3.54	1.00	1.45	0.19	1.37	1.16

*, **Significant at 0.05 and 0.01 probability level. [1]DF: Degrees of freedom. [2]Treatments: seaweed extract, humic acid, and potassium sulfate. [3]LSD: Least significant difference at $\alpha = 0.05$.

following the application of humic acid, whereas under the higher salt level (15 dS·m^{-1}), the application of seaweed extract resulted in the lowest Na/K ratio (Table 7). The lower Na/K ratio in the salt-tolerant genotype is a possible indication of higher Na exclusion in this genotype. The higher content of Ca in the two genotypes under both salt concentrations 10 and 15 dS·m^{-1}, and under all the growth enhancing treatments compared to the no-salt control was possibly attributable to the fact that calcium was one of the components of the saline solution. Magnesium, P, N, S, Fe and B concentrations were reduced in both genotypes with increasing salt levels, and Zn, Mn, Cu and Al were slightly increased (Table 7). There were significant interactions ($p < 0.01$) between genotypes and salt levels for Ca, N, Mn, Al and B. The application of potassium sulfate to the salt-sensitive genotype resulted in the highest plant uptake of macro/micro elements under 10 dS·m^{-1} compared the other two growth enhancing treatments. Alfalfa growth and development depends upon the availability of adequate amounts of potassium, which increases crops overall productivity [41]. Similar results of the effect of potassium sulfate application under salt stress were reported in wheat [42]. Application of seaweed extract to the salt-tolerant genotype boosted the accumulation of Mg, P, N, S, Fe, Zn, Mn, Cu, B and Al in plant tissue under both salt levels (10 and 15 dS·m^{-1}). Similar observations were reported following the foliar application of seaweed extract on alfalfa plants at a rate of 300 g, which resulted in increased plant macro/micro elements such as N, P, K, Fe, Mn, and Zn [43]. These results suggest that seaweed extract may have the ability to improve stress tolerance [44] [45] by increasing nutrient uptake from the soil [46]. The beneficial effect of seaweed extract may be the result of many components working synergistically at different concentrations [47] especially those derived from *Ascophyllum nodosum*, which were reported to have

Table 7. Average mineral composition of plant tissue of two Alfalfa genotypes evaluated under three salt levels (0, 10, and 15 dS·m^{-1} electrical conductivity) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Genotypes	Salt level	Treatments	Na/K	Ca	Mg	P	N	S	Fe	Zn	Mn	Cu	B	Al
				g·Kg^{-1}					mg·Kg^{-1}					
Bulldog 505	0 dS·m^{-1}	Control (0 salt)	0.4 ± 0.01	12.3 ± 0.1	6.7 ± 1.0	2.4 ± 0.02	33.9 ± 0.4	2.5 ± 0.1	58.9 ± 5.1	19.1 ± 0.7	33.7 ± 0.9	2.7 ± 0.1	63.2 ± 3.0	13.8 ± 0.7
	10 dS·m^{-1}	Seaweed extract	17.3 ± 2.9	33.4 ± 3.0	1.8 ± 0.2	1.7 ± 0.01	29.2 ± 0.2	2.1 ± 0.1	40.7 ± 0.3	25.8 ± 1.7	33.4 ± 2.5	2.7 ± 0.2	53.6 ± 2.2	10.0 ± 0.0
		Humic acid	14.9 ± 2.4	32.8 ± 0.0	1.6 ± 0.1	1.8 ± 0.01	29.8 ± 0.9	2.1 ± 0.1	37.6 ± 0.3	23.9 ± 1.3	44.5 ± 0.4	4.1 ± 0.1	52.3 ± 0.9	11.3 ± 2.3
		Potassium sulfate	12.8 ± 0.6	36.0 ± 2.0	1.6 ± 0.1	2.1 ± 0.01	30.4 ± 0.2	2.1 ± 0.1	49.6 ± 0.3	25.6 ± 0.4	47.4 ± 0.6	5.1 ± 0.1	56.4 ± 2.3	10.3 ± 0.6
	15 dS·m^{-1}	Seaweed extract	24.2 ± 0.5	34.0 ± 1.0	1.4 ± 0.1	2.1 ± 0.02	30.5 ± 0.4	2.2 ± 0.2	77.8 ± 2.3	32.4 ± 3.0	47.9 ± 0.3	5.4 ± 0.4	58.4 ± 2.3	50.5 ± 5.3
		Humic acid	22.3 ± 5.6	43.0 ± 1.0	1.2 ± 0.2	2.2 ± 0.02	30.3 ± 0.2	2.3 ± 0.0	41.5 ± 1.9	25.0 ± 2.2	44.9 ± 1.6	5.3 ± 0.3	59.4 ± 3.5	10.0 ± 0.0
		Potassium sulfate	20.4 ± 1.4	40.0 ± 0.2	1.5 ± 0.2	2.2 ± 0.02	29.9 ± 1.9	2.5 ± 0.2	46.9 ± 1.5	26.5 ± 0.8	44.4 ± 3.1	5.8 ± 0.1	58.5 ± 1.6	23.3 ± 1.5
Mesa sirsa	0 dS·m^{-1}	Control (0 salt)	0.4 ± 0.02	18.0 ± 0.4	5.6 ± 0.1	2.3 ± 0.03	31.3 ± 0.2	3.1 ± 0.2	62.6 ± 0.7	17.5 ± 1.1	57.2 ± 1.9	4.1 ± 0.2	75.1 ± 3.3	11.4 ± 1.0
	10 dS·m^{-1}	Seaweed extract	15.1 ± 0.8	37.0 ± 1.0	1.7 ± 0.2	2.1 ± 0.005	28.1 ± 0.4	2.5 ± 0.1	51.03 ± 5.2	30.8 ± 2.2	47.5 ± 1.8	4.6 ± 0.3	54.2 ± 0.3	10.0 ± 0.0
		Humic acid	14.6 ± 0.3	37.0 ± 6.0	1.4 ± 0.1	1.9 ± 0.01	27.4 ± 0.7	2.1 ± 0.2	42.9 ± 3.1	19.7 ± 0.1	43.0 ± 4.5	4.1 ± 0.5	48.8 ± 1.3	10.0 ± 0.0
		Potassium sulfate	20.2 ± 1.0	42.0 ± 1.0	1.6 ± 0.2	1.8 ± 0.01	26.6 ± 0.6	2.0 ± 0.1	38.6 ± 1.7	23.5 ± 1.3	45.3 ± 2.9	4.0 ± 0.2	46.8 ± 2.2	10.0 ± 0.0
	15 dS·m^{-1}	Seaweed extract	25.4 ± 3.8	37.0 ± 1.0	1.3 ± 0.1	2.3 ± 0.01	30.8 ± 0.8	2.5 ± 0.2	66.3 ± 0.9	28.2 ± 0.7	47.2 ± 2.2	5.7 ± 0.6	54.8 ± 2.2	27.1 ± 1.1
		Humic acid	32.1 ± 2.7	41.0 ± 4.0	1.2 ± 0.1	2.1 ± 0.02	30.10.2	2.1 ± 0.1	51.7 ± 5.5	26.0 ± 2.6	42.2 ± 3.7	3.6 ± 0.2	46.1 ± 1.9	28.7 ± 3.2
		Potassium sulfate	26.7 ± 3.9	37.0 ± 1.0	1.2 ± 0.0	2.3 ± 0.01	31.1 ± 0.2	2.3 ± 0.2	62.0 ± 18.2	27.3 ± 0.9	48.3 ± 0.9	5.9 ± 0.5	54.0 ± 0.6	10.0 ± 0.0

high concentrations of total phenolics, a complex group known for its strong chelating activities [48] [49].

3.2.3. Plant Biomass

Salt concentrations had significant effects ($p < 0.01$) on shoot and root dry weights. Plant biomass was reduced under both salt concentrations in both genotypes, with a greater magnitude in the salt-sensitive Bulldog 505, compared with the no-salt control (**Table 9**). Growth reduction in beans (*Phaseolus vulgaris*) treated with 50 and 100 mM sodium chloride were reported [50]. Barley plants exposed to salt levels of 0.75 and 13 $dS{\cdot}m^{-1}$ with and without potassium application also showed a similar response [51] and so did wheat plants under salt stress [42]. There was a significant difference ($p < 0.01$) between the two alfalfa genotypes in their response to salt levels and growth-stimulant treatments applied (**Table 8**). Application of seaweed extract to the salt sensitive genotype resulted in higher shoot and root dry weights compared with the other treatments under 10 $dS{\cdot}m^{-1}$ recording, 1.68 and 2.97 g, respectively (**Table 9**). The application of sea algae extracts) as organic bio-stimulants is becoming an accepted practice in horticulture industry, helping plants becoming more disease resistant and enabling rapid root development [52]. At the higher salt level (15 $dS{\cdot}m^{-1}$), the salt sensitive genotype responded with higher shoot and root dry weights following the application of K_2SO_4 nanoparticles. Adequate availability of potassium increased dry mass production of plants under salt and drought stress compared to lower concentrations of potash fertilizer [41]. The addition of humic acid increased plant biomass of the salt-tolerant genotype Mesa-Sirsa under both 10 and 15 $dS{\cdot}m^{-1}$ compared with the other treatments (**Table 9**). The increased growth observed following the application of humic acid might be attributed to its positive effects on the soil, which led to an increase in CEC and

Table 8. Mean squares and significance of shoot shoot dry weight (gm), plant height (cm), root length (cm), and root dry weight (gm) in two alfalfa genotypes grown under three salt levels (0, 10 and 15 $dS{\cdot}m^{-1}$ EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Source of variation	Degrees of freedom	Shoot dry weight	Plant height	Root length	Root dry weight
Genotype	1	0.1*	1.3	168.7*	3.6**
Salt level	1	1.01**	32.4**	0.1	1.8*
Genotype × Salt level	1	0.001	15.3*	12.5	0.004
Treatments	2	0.1*	4.9	0.04	0.004
Genotype × Treatments	2	0.1*	7.51	25.0	1.8*
Salt level × Treatments	2	0.02	0.4	0.2	0.1
Genotype × Treatments × Salt levels	2	0.2**	19.3**	5.5	0.1
Error	28	0.02	2.6	23.8	0.3
LSD		0.1	1.02	3.1	0.3

*, **Significant at 0.05 and 0.01 probability level.

Table 9. Shoot dry weight (gm), plant height (cm), root length (cm), and root dry weight (gm) of two alfalfa genotypes grown under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Genotype	Salt level	Treatments	Shoot dry weight (g)	Plant height (cm)	Root length (cm)	Root dry weight (g)
	Control (0 salt)	Control (0 salt)	2.49 ± 0.18	32.38 ± 1.68	29.88 ± 7.65	3.67 ± 0.49
		Seaweed extract	1.68 ± 0.20	19.86 ± 0.91	21.05 ± 2.51	2.97 ± 0.71
	10 dS·m^{-1}	Humic acid	1.67 ± 0.11	22.35 ± 2.15	20.22 ± 4.54	2.04 ± 0.15
Bulldog 505		Potassium sulfate	1.40 ± 0.11	20.45 ± 1.91	23.33 ± 6.78	2.92 ± 0.60
		Seaweed extract	1.19 ± 0.10	19.78 ± 1.51	18.94 ± 2.46	2.24 ± 0.59
	15 dS·m^{-1}	Humic acid	1.16 ± 0.15	18.66 ± 2.47	20.49 ± 10.01	1.84 ± 0.50
		Potassium sulfate	1.42 ± 0.03	22.44 ± 1.39	21.96 ± 7.12	2.44 ± 0.93
	Control (0 salt)	Control (0 salt)	2.02 ± 0.11	27.11 ± 2.07	20.32 ± 3.91	2.34 ± 0.39
		Seaweed extract	1.47 ± 0.14	24.26 ± 0.42	18.28 ± 1.47	2.08 ± 0.47
	10 dS·m^{-1}	Humic acid	1.76 ± 0.04	24.57 ± 2.28	19.18 ± 2.83	2.67 ± 0.44
Mesa-Sirsa		Potassium sulfate	1.56 ± 0.34	24.51 ± 0.71	16.40 ± 2.65	2.05 ± 0.44
		Seaweed extract	1.23 ± 0.09	20.41 ± 1.88	21.16 ± 2.52	1.67 ± 0.12
	15 dS·m^{-1}	Humic acid	1.49 ± 0.10	23.82 ± 0.47	19.05 ± 1.67	2.29 ± 0.16
		Potassium sulfate	1.03 ± 0.07	19.50 ± 0.57	17.51 ± 1.66	1.55 ± 0.30

nutrients (Table 5). Humic acid resulted in higher rates of K uptake and low Na/K ratio (Table 7), which may lead to overcoming the stress induced by alterations in the balance of endogenous hormones. Humic substances might exhibit anti-stress effects under abiotic stress conditions such as salinity stress [18] [53] [54]. It has been suggested that humic acid increases root cell elongation by acting on growth hormones, resulting in increased cell membrane permeability, oxygen uptake, respiration, and photosynthesis [55] [56] [57] [58].

3.2.4. Physiological Responses

1) Relative water content (RWC)

Relative water content is an index describing the amount of water in plant tissue and indicates the ability of a plant to maintain adequate water under stress conditions [59]. There was a significant effect ($p < 0.01$) of salt concentrations on RWC. In both genotypes, RWC was lower under 10 and 15 dS·m^{-1} compared with the no-salt control, regardless of the growth-stimulant compound applied, and decreased with increasing salt level (Table 11). Similar reports were reported by Chen *et al.* [60]. Application of plant growth stimulants to the two alfalfa genotypes under the high salt levels affected significantly ($p < 0.01$) plant relative water content (Table 10). In the salt sensitive genotype Bulldog, RWC was higher following the application of seaweed extract compared with humic acid and potassium sulfate treatments under both salt levels 10 and 15 dS·m^{-1} (Table 11). Seaweed extracts are known to contain osmolytes such as mannitol, an important protectant compound under abiotic stress [12] that enhances root growth as well as soil water-holding capacity [11]. In the salt tolerant genotype,

Table 10. Means squares of relative water content (RWC), electrolyte leakage (EL) and proline in response to the application of three growth-enhancing treatments (seaweed extract, humic acid, and potassium sulfate) to alfalfa genotypes grown under two salt levels (10 and 15 dS·m^{-1} electrical conductivity).

Source of variation	Degrees of Freedom	RWC	Electrolyte leakage	Proline
Genotype	1	15.71	1358.73**	1.17**
Salt level	1	116.17**	24.24**	1.31**
Genotype × Salt level	1	119.65**	24.59**	0.75*
Treatments	2	47.22**	275.42**	0.11
Genotype × Treatments	2	41.99**	340.77**	2.71**
Salt level × Treatments	2	110.31**	219.74**	3.96**
Genotype × Treatments × Salt levels	2	37.33**	239.85**	1.86**
Error	28	4.02	2.39	0.166
LSD		1.27	0.98	0.26

*, **Significant at 0.05 and 0.01 probability level.

Table 11. Relative water content (RWC), Proline, and Electrical leakage (EL) in two alfalfa genotypes grown under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Genotype	Salt level	Treatments	RWC (%)	Electrolyte leakage (%)	Proline (μmol·g^{-1} FW)
Bulldog 505	Control (0 salt)	Control (0 salt)	48.10 ± 0.41	51.03 ± 3.32	2.90 ± 0.74
	10 dS·m^{-1}	Seaweed extract	43.51 ± 0.63	63.29 ± 0.53	2.08 ± 0.87
		Humic acid	43.47 ± 3.2	81.84 ± 0.73	2.27 ± 0.04
		Potassium sulfate	31.65 ± 0.10	76.40 ± 1.34	3.16 ± 0.30
	15 dS·m^{-1}	Seaweed extract	42.74 ± 0.53	65.57 ± 1.78	3.61 ± 0.50
		Humic acid	34.43 ± 1.29	69.50 ± 1.01	1.98 ± 0.49
		Potassium sulfate	41.63 ± 0.85	80.43 ± 1.14	2.20 ± 0.36
Mesa-Sirsa	Control (0 salt)	Control (0 salt)	46.28 ± 0.07	74.45 ± 0.87	1.83 ± 0.14
	10 dS·m^{-1}	Seaweed extract	44.71 ± 1.21	81.43 ± 0.22	1.58 ± 0.21
		Humic acid	46.03 ± 3.53	87.96 ± 1.69	2.06 ± 0.21
		Potassium sulfate	45.77 ± 5.05	87.46 ± 0.24	2.67 ± 0.20
	15 dS·m^{-1}	Seaweed extract	34.42 ± 0.22	75.85 ± 1.86	2.44 ± 0.17
		Humic acid	39.66 ± 1.79	81.35 ± 1.45	4.12 ± 0.25
		Potassium sulfate	40.71 ± 0.38	83.57 ± 2.27	1.76 ± 0.27

Mesa-Sirsa, the highest RWC was achieved by the addition of humic acid under 10 dS·m^{-1} (46.03%) and K_2SO_4 nanoparticles under 15 dS·m^{-1} (40.71%). The in-

crease in the RWC following the application of humic acid may be attributed to enhanced root biomass (root length and dry weight) that leads to absorbing more water under salt stress, while potassium acts as an osmo-regulator in the cell [61] [62] [63].

2) Electrolyte leakage

Electrolyte leakage (EL) is an indirect assessment of the degree of cell membrane integrity [64], and is considered a reliable physiological indicator of the degree of plant cell injury under stress conditions [65]. Increasing salt concentrations resulted in significant increases ($p < 0.01$) in EL in both genotypes compared to the no-salt control (**Table 10**). Application of plant growth stimulants to the two alfalfa genotypes resulted in significantly different ($p < 0.01$) responses in electrolyte leakage under the two salt concentrations. Application of seaweed extract enhanced the response of both alfalfa genotypes to salt stress by decreasing EL at both salt levels, 10 and 15 $dS \cdot m^{-1}$ (**Table 11**). Seaweed extract was reported to significantly improve water use efficiency, increase leaf water content, and improve salt stress tolerance [66] [67].

3) Proline

Proline accumulation is a sensitive physiological index of plant response to various abiotic stresses [68]. It plays a positive role in the salt tolerance of many crops by contributing to membrane stability [69]. Proline also induces the expression of salt stress-responsive proteins and may improve the plant adaptation to salt stress [70]. There was a significant difference ($p < 0.01$) between the alfalfa genotypes in proline accumulation in plant tissue in response to salt concentrations. In the salt sensitive genotype bulldog, the application of potassium sulfate resulted in the highest increase in proline content with 3.16 $\mu mol \cdot g^{-1}$ FW under 10 $dS \cdot m^{-1}$ (**Table 11**), while seaweed extract resulted in the highest increase (3.61 $\mu mol \cdot g^{-1}$ FW) under 15 $dS \cdot m^{-1}$ compared to 2.90 $\mu mol \cdot g^{-1}$ FW in the no-salt control. In the salt tolerant genotype Mesa-Sirsa, the highest proline accumulation was observed following the application of potassium sulfate at the 10 $dS \cdot m^{-1}$ (2.67 $\mu mol \cdot g^{-1}$ FW) and following the application of humic acid under 15 $dS \cdot m^{-1}$ (4.12 $\mu mol \cdot g^{-1}$ FW) compared to 1.83 $\mu mol \cdot g^{-1}$ FW in the no-salt control (**Table 11**).

4) Proline

Proline accumulation is a sensitive physiological index of plant response to various abiotic stresses [68]. It plays a positive role in the salt tolerance of many crops by contributing to membrane stability [69]. Proline also induces the expression of salt stress-responsive proteins and may improve the plant adaptation to salt stress [70]. There was a significant difference ($p < 0.01$) between the alfalfa genotypes in proline accumulation in plant tissue in response to salt concentrations. In the salt sensitive genotype bulldog, the application of potassium sulfate resulted in the highest increase in proline content with 3.16 $\mu mol \cdot g^{-1}$ FW under 10 $dS \cdot m^{-1}$ (**Table 11**), while seaweed extract resulted in the highest increase (3.61 $\mu mol \cdot g^{-1}$ FW) under 15 $dS \cdot m^{-1}$ compared to 2.90 $\mu mol \cdot g^{-1}$ FW in the no-salt control. In the salt tolerant genotype Mesa-Sirsa, the highest proline ac-

cumulation was observed following the application of potassium sulfate at the 10 $dS \cdot m^{-1}$ (2.67 $\mu mol \cdot g^{-1}$ FW) and following the application of humic acid under 15 $dS \cdot m^{-1}$ (4.12 $\mu mol \cdot g^{-1}$ FW) compared to 1.83 $\mu mol \cdot g^{-1}$ FW in the no-salt control (Table 11).

3.2.5. Antioxidant Enzymes

There was a significant difference ($p < 0.01$) between the genotypes in their CAT activity following the increase in salt concentrations and the application of growth stimulant treatments (Table 12). Increasing salt concentration resulted in significant increases ($p < 0.01$) in CAT activity in both genotypes compared to the no-salt control. (Table 12, Figure 1). Application of growth stimulants resulted in significant increases ($p < 0.01$) in CAT activity in both genotypes. Application of seaweed extract resulted in the highest increase of CAT in both genotypes under 10 $dS \cdot m^{-1}$ salt level and was three times higher than the control in the salt sensitive bulldog and four times higher than the control in the salt tolerant Mesa-Sirsa. Under the 15 $dS \cdot m^{-1}$ salt level, seaweed extract also resulted in the highest CAT activity in the salt tolerant genotype and was similar to humic acid in the salt sensitive genotype (Figure 1). Application of humic acid and potassium sulfate resulted in no change in CAT activity at 10 $dS \cdot m^{-1}$ in both genotypes compared to the no-salt control. However, they resulted in steep increases in CAT in both genotypes under 15 $dS \cdot m^{-1}$ salt level (Figure 1). A similar trend was observed for SOD activity. The application of growth stimulants resulted in significant increases ($p < 0.01$) in SOD in both genotypes at both salt levels compared to the no-salt control (Table 12). The response of the genotypes to the application of growth stimulants was similar under both salt levels. The application of seaweed extract resulted in the highest increase in SOD activity at the 10 $dS \cdot m^{-1}$ salt level in both genotypes, and was 92% higher in the bulldog genotype and 16% higher in the Mesa-Sirsa genotype compared to the control (Figure 2).

Table 12. Means squares of catalase (mmol H_2O_2 $min^{-1} \cdot g^{-1}$ fresh tissue) and SOD (Super oxide dismutase in $U \cdot g^{-1}$ fresh tissue) in two alfalfa genotypes grown under three salt levels (0, 10 and 15 $dS \cdot m^{-1}$ EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Source of variation	DF	Catalase	SOD
Genotype	1	1589.75**	51.91
Salt level	1	1664.23**	25.39
Genotype × Salt level	1	97.19**	109.51
Treatments	2	1839.25**	811.36**
Genotype × Treatments	2	163.70**	355.18*
Salt level × Treatments	2	964.24**	697.71**
Genotype × Treatments × Salt levels	2	436.50**	261.06**
Error	28	5.73	77.94
LSD		1.51	5.58

*, **Significant at 0.05 and 0.01 probability level.

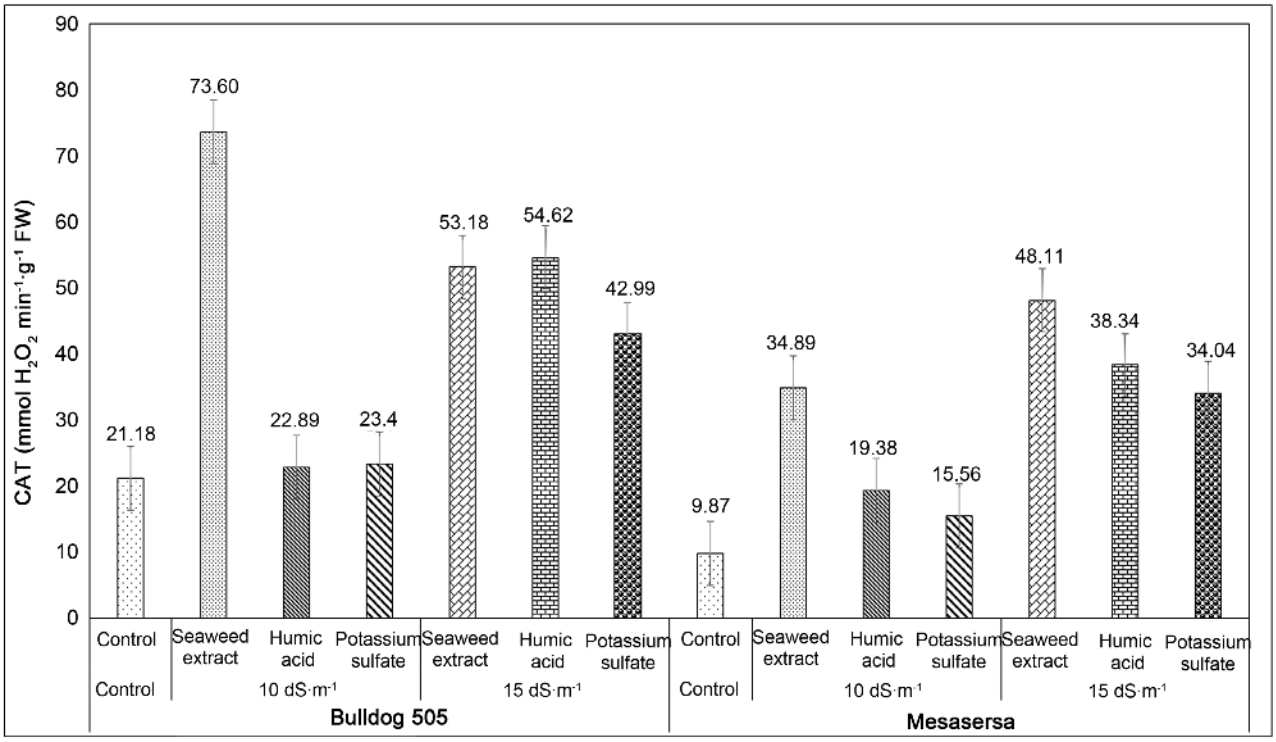

Figure 1. Catalase activity (mmol H_2O_2 min^{-1}·g^{-1} fresh tissue) in one salt sensitive (Bulldog) and one salt tolerant (Mesa-Sirsa) alfalfa genotypes grown under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

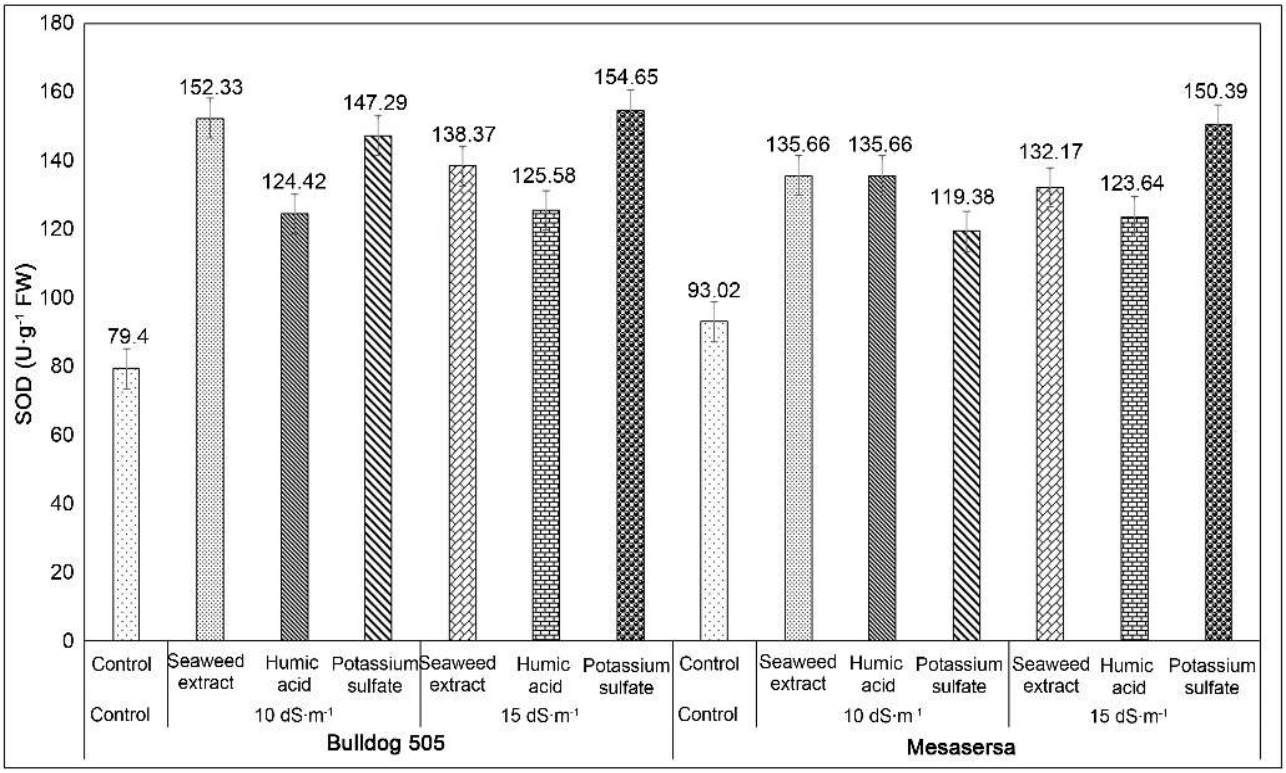

Figure 2. Superoxide dismutase (U·g^{-1} fresh tissue) in one salt sensitive (Bulldog) and one salt tolerant (Mesa-Sirsa) alfalfa genotypes grown under three salt levels (0, 10 and 15 dS·m^{-1} EC) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

Potassium sulfate resulted in the highest increases in SOD activity in both genotypes under the 15 $dS \cdot m^{-1}$ salt level, but was not much different from seaweed extract (**Figure 2**). Seaweed extract is known to contain betaines, including gamma-aminobutyric acid betaine, 6-aminovaleric acid betaine, and glycine betaine, which play an important role in enhancing chlorophyll and antioxidant enzymes [71].

There is evidence that seaweed extract increases tolerance to oxidative stress and protects against adverse environmental conditions by enhancing the activity of the antioxidant enzymes SOD and Ascorbate peroxidase (ASP) [16] [72]. Potassium plays a key role in plants through the activation of enzymes by stabilizing the pH between 7 and 8 and changing the conformation of enzymes by binding to their surfaces [73]. Tripathi *et al.* [74] reported that proteins, such as thioredoxin, glutaredoxin, and cyclophilin, facilitated the regeneration of the catalytically active form of peroxiredoxins that played an important role in reducing the formation of reactive oxygen species in plants under biotic and abiotic stress.

3.3. Correlation between Physiological and Phenotypic Responses

There was a significant positive correlation ($r = 0.42$, $p < 0.01$) between proline content in the plant tissue and relative water content (**Table 13**). Proline content was also negatively correlated ($r = -0.18$, $p < 0.05$) with electrolyte leakage. Proline plays important roles as an osmo-protectant and acts as an antioxidant as

Table 13. Correlation between shoot dry weight (g), plant height (cm), root length (cm), root dry weight (g), relative water content (%), electrolyte leakage (%), Proline ($\mu mol \cdot g^{-1}$ FW), of two alfalfa genotypes evaluated under three salt levels (0, 10, and 15 $dS \cdot m^{-1}$ electrical conductivity) and three treatments of growth-enhancing substances (seaweed extract, humic acid, and potassium sulfate).

	Shoot dry weight	Plant height	Root length	Root dry weight	Relative Water Content	Electrolyte leakage	Proline	CAT	SOD
Shoot dry weight	1.00	0.76**	0.24	0.62**	0.57**	−0.03	0.003	0.34*	0.50**
Plant height	-	1.00	0.2	0.35	0.57**	−0.07	−0.04	0.37*	0.55**
Root length	-	-	1.00	0.44**	0.04	−0.29	0.05	0.05	−0.08
Root dry weight	-	-	-	1.00	0.19	−0.08	0.19	0.22	0.17
Relative Water Content	-	-	-	-	1.00	−0.04	0.42**	−0.28	0.44**
Electrolyte leakage	-	-	-	-	-	1.00	−0.18	−0.40**	0.33
Proline	-	-	-	-	-	-	1.00	0.14	0.17
CAT	-	-	-	-	-	-	-	1.00	0.47**
SOD	-	-	-	-	-	-	-	-	1.00

well as a source of energy [75] [76]. It is generally considered an important biomolecule that has a protective role in tolerance of plant to abiotic stresses [77] [78] leading to a higher water potential, and hence improving the water uptake and growth under stress [79]. This explains the negative correlation observed in this study between proline and electrolyte leakage and the positive correlation with relative water content. The accumulation of proline under stress is an adaptation mechanism, regulating membrane water permeability in cells and influencing water movement among tissue and organs [80], leading to enhanced plant growth. This may explain the positive correlations observed between RWC and shoot dry weight ($r = 0.57$, $p < 0.01$) and the positive correlation ($r = 0.62$, $p < 0.01$) between shoot dry weight and plant root dry weight. Catalase activity showed a significant negative correlation with electrolyte leakage ($r = -0.40$, $p < 0.01$) and significant positive correlations with shoot dry weight ($r = 0.34$, $p < 0.05$) and plant height ($r = 0.37$, $p < 0.05$). SOD activity showed positive correlations with shoot dry weight ($r = 0.50$, $p < 0.01$) and plant height ($r = 0.55$, $p < 0.01$), but a low correlation with root dry weight ($r = 0.17$, $p > 0.05$). It also showed a significant positive correlation with relative water content ($r = 0.44$, $p < 0.01$). The significant positive correlation between SOD and CAT activity ($r = 0.47$, $p < 0.01$) is an indication that the two enzymes act synergistically to enhance alfalfa tolerance to salt stress.

4. Conclusion

Increasing salt concentration reduced germination in all alfalfa genotypes. Above 1% salt in the solution reduced seed germination by more than 70% in most genotypes. Growing alfalfa plants under salt concentration of 10 $dS \cdot m^{-1}$ and above, resulted in significant decreases in plant shoot and root growth, with a lesser magnitude in salt tolerant genotypes. Application of plant growth stimulants (seaweed extract, humic acid, and potassium sulfate) to one salt tolerant and one salt sensitive genotype grown under two salt levels (10 and 15 $dS \cdot m^{-1}$) resulted in overall changes in soil properties relative to the no-salt control. Application of humic acid improved the growth of the salt-tolerant genotype under both salt concentrations, while seaweed extract and potassium sulfate were more effective in the salt sensitive genotype under 10 and 15 $dS \cdot m^{-1}$. Overall, potassium sulfate was more effective in maintaining a low Na/K ratio in the susceptible genotype under 10 $dS \cdot m^{-1}$ and humic acid under 15 $dS \cdot m^{-1}$, while in the salt tolerant genotype, humic acid was more effective in maintaining a low Na/K ratio under 10 $dS \cdot m^{-1}$ and seaweed extract under 15 $dS \cdot m^{-1}$. Seaweed extract resulted in higher RWC and proline under both salt concentrations (10 and 15 $dS \cdot m^{-1}$) in the salt sensitive genotype and lower electrolyte leakage in both salt tolerant and salt sensitive genotypes under both salt concentrations. Seaweed extract also enhanced CAT and SOD activities in both genotypes under 10 $dS \cdot m^{-1}$. Application of seaweed extract seemed to have an overall positive effect in alleviating salt stress in the salt-sensitive alfalfa genotype, while the application of humic acid and potassium sulfate seemed to have an overall positive effect in maintaining

growth under salt stress in the salt-tolerant alfalfa genotype.

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