Volume 14, Issue 3, Page 172-182, 2025 https://doi.org/10.46810/tdfd.1708150 Research Article

BİNGÖL UNIVERSITY

Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

Combined Humic Acid and Lichen-Derived Endophytic Bacteria Application Alleviates Salt Stress-Induced Oxidative Damage in Alfalfa (*Medicago sativa* L.)

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(Received: 28.05.2025, Accepted: 03.09.2025, Online Publication: 26.09.2025)

Keywords
Salt stress, Alfalfa, Humic acid, Endophyte, Antioxidant, Lichen,

Abstract: This study investigates the synergistic effects of leonardite-sourced humic acid (HA) and a lichen-derived endophytic bacterium (*Bacillus wiedmannii*) in mitigating salt stress in alfalfa (*Medicago sativa* L., cv. Konya). Alfalfa seeds were grown in a peat-perlite mix with different combinations of 100 mM NaCl, HA (500 or 1000 mg L^{-1}), and *B. wiedmannii*. After 35 days, relative water content (RWC), ROS (H_2O_2, $O_2^{\cdot-}$), MDA, antioxidant enzyme activities, and photosynthetic pigments were measured. The salt stress alone reduced RWC, the photosynthetic pigment amount, and the antioxidant enzyme activities, while elevating H_2O_2 and $O_2^{\cdot-}$, and MDA accumulation. HA or *B. wiedmannii* (Bw) individually partially ameliorated these effects, however, the combined HA+Bw treatment under saline conditions (especially 1000 mg L^{-1} HA+Bw+NaCl) elevated RWC, and reduced ROS and MDA levels close to the control values. In addition, the antioxidant enzyme activities and the photosynthetic pigment contents were enhanced under the combined treatments compared to the salt alone. These results demonstrate a synergistic effect between HA and *B. wiedmannii* in alleviating salt stress in the alfalfa by enhancing water status, reducing oxidative damage, and activating antioxidant defenses, suggesting that the co-application of HA and *B. wiedmannii* is a promising strategy for improving salt tolerance in forage crops.

Humik Asit ve Liken Türevli Endofitik Bakterilerin Kombine Uygulaması, Yoncada (*Medicago sativa* L.) Tuz Stresi Kaynaklı Oksidatif Hasarı Yatıştırır

Anahtar Kelimeler
Tuz stresi, Yonca, Humik asit, Endofit, Antiosidan, Liken,

Öz: Bu çalışma, leonardit kaynaklı humik asit (HA) ile liken kökenli bir endofitik bakteri (*Bacillus wiedmannii*) arasındaki sinerjik etkinin, yonca bitkisinde (*Medicago sativa* L., cv. Konya) tuz stresini hafifletmedeki rolünü araştırdı. Yonca tohumları, 100 mM NaCl, HA (500 veya 1000 mg L^{-1}) ve *B. wiedmannii*'nin farklı kombinasyonlarını içeren turba-perlit karışımında ekildi. 35 gün sonra, bağıl su içeriği (RWC), reaktif oksijen türleri (H_2O_2, $O^{\cdot-}$), MDA (malondialdehit), antioksidan enzim aktiviteleri ve fotosentetik pigment düzeyleri ölçülmüştür. Tuz stresi, tek başına uygulandığında RWC, fotosentetik pigment miktarı ve antioksidan enzim aktivitelerinde azalmaya, H_2O_2, $O^{\cdot-}$, ve MDA birikiminde ise artışa neden olmuştur. HA veya *B. wiedmannii* tek başına bu olumsuz etkileri kısmen hafifletmiştir. Ancak, özellikle 1000 mg L^{-1} HA+*B. wiedmannii*+NaCl kombinasyonu, RWC'yi artırmış ve ROS ile MDA seviyelerini kontrol grubuna yakın düzeylere düşürmüştür. Ayrıca, bu kombine uygulama antioksidan enzim aktiviteleri ile fotosentetik pigment içeriklerini, sadece tuz uygulamasına kıyasla artırmıştır. Bu sonuçlar, HA ile *B. wiedmannii* arasındaki sinerjik etkinin, su dengesini iyileştirerek, oksidatif zararı azaltarak ve antioksidan savunma sistemlerini aktive ederek tuz stresini hafiflettiğini göstermektedir. Bu nedenle HA ve *B. wiedmannii*'nin birlikte uygulanması, yem bitkilerinde tuz toleransını artırmak için umut verici bir strateji olarak değerlendirilebilir.

1. INTRODUCTION

Salt (NaCl) stress is one of the major abiotic stress factors threatening agricultural production worldwide. In particular, in irrigation-based agricultural systems, the accumulation of Na^+ and Cl^- ions in the soil hinders water uptake by plants and disrupts ionic balance by interfering with the absorption of essential nutrients such as potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) [1, 2]. Salt stress also causes excessive accumulation of reactive oxygen species (ROS), leading to oxidative damage to proteins, lipids, DNA, and cellular membranes. ROS components such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$) are produced abundantly in energy-related organelles such as chloroplasts, mitochondria, and peroxisomes, thereby threatening cellular integrity. Plants attempt to neutralize these harmful compounds through non-enzymatic antioxidants and enzymatic such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). However, under severe stress conditions, these defense mechanisms may prove insufficient [3, 4]. Consequently, cell membrane damage, reduced photosynthetic activity, stunted growth, and disruption of metabolic processes may occur. Soil salinity affects not only plant health but also the microbial structure of the soil. High salt concentrations reduce the activity and populations of beneficial microorganisms, thereby weakening their symbiotic relationships with plants. Combined with physical problems such as soil structure deterioration, reduced water permeability, and hindered aeration, this leads to a further decline in productivity [1, 5].

Humic acid (HA), a natural biostimulant, is gaining attention in environmentally friendly agricultural practices. HA is complex substances formed during the decomposition of organic matter and are found in soil, water, and sediments [6, 7]. Stabilized by hydrophobic interactions and hydrogen bonds, these compounds exert multifaceted effects such as regulating osmotic balance, maintaining ion homeostasis, supporting photosynthesis, and reducing oxidative stress. They also promote the accumulation of osmolytes like proline to reduce water loss and limit Na^+ accumulation while facilitating the uptake of beneficial ions such as K^+, Ca^{2+}, and Mg^{2+} [8, 9, 10]. Additionally, HA enhances chlorophyll synthesis and activate antioxidant defense systems to maintain cellular integrity [7]. Their ability to support microbial viability and facilitate ion exchange makes them valuable components in biofertilizer formulations. By promoting soil aggregation, increasing water retention capacity, regulating pH, and enhancing cation exchange capacity, HA helps create a more favorable microhabitat in the soil [11]. This environment facilitates colonization by microorganisms-especially fungi and bacteria-in the rhizosphere. However, the potential synergistic or carrier role of HA in conjunction with beneficial microorganisms remains underexplored.

Recent limited studies have highlighted the promising role of lichen-derived microorganisms in plant stress responses, drawing growing interest in their use in agricultural biotechnology [12, 13, 14]. Lichens, which are symbiotic organisms composed of algae/cyanobacteria and fungi, offer metabolic flexibility and microbial diversity that can support ion balance, nitrogen fixation, phosphate solubilization, and oxidative stress mitigation [14, 15]. These microorganisms also support plants through mechanisms such as the production of phytohormones like indole-3-acetic acid (IAA), gibberellins, and cytokinins; secretion of ACC deaminase to reduce the stress hormone ethylene; siderophore production to enhance iron uptake; nitrogen fixation; and increased phosphate solubilization [16, 17]. Some endophytic microorganisms isolated from lichens have also demonstrated plant growth-promoting effects similar to those of plant growth-promoting rhizobacteria (PGPR), reducing ion toxicity and effectively mitigating osmotic stress [12]. Nevertheless, studies examining the combined use of HA and lichen-derived microorganisms on forage crops of economic importance are still quite limited.

Alfalfa (*Medicago sativa* L.) is a valuable forage crop due to its high protein, mineral, and vitamin content, and also stands out for its contribution to biological nitrogen fixation and soil fertility. Alfalfa not only serves as a fundamental feed source in livestock production but is also appreciated for its high biomass yield, wide adaptation capacity, natural resistance to diseases, ability to improve soil structure, and ecosystem services [18, 19]. However, environmental stress factors, particularly salt stress, negatively affect key physiological processes in alfalfa, such as germination, photosynthesis, and growth, leading to yield loss. Alfalfa is especially sensitive to salt stress during the germination and early seedling stages. Under saline conditions, physiological responses such as stomatal closure, chlorophyll degradation, ion accumulation, and a decline in turgor pressure significantly restrict alfalfa development and productivity [4, 20].

This study hypothesizes that the HA can alleviate salt-induced damage in plants by modulating the antioxidant system, and that lichen-derived microorganisms may synergistically enhance this effect by reducing oxidative stress through the secretion of antioxidant compounds. In line with this hypothesis, the study investigates the combined application of the leonardite-derived HA and an endophytic bacterium (*Bacillus wiedmannii*) isolated from the lichen *Rhizoplaca melanophthalma*, and evaluates their effects on the physiological and biochemical responses of alfalfa (*Medicago sativa* L., cv. Konya) under salt stress conditions.

2. MATERIALS AND METHODS

2.1. Determination of Salt and Humic Acid Concentrations

In this study, alfalfa (*Medicago sativa* L., cv. Konya) was obtained from the Eastern Anatolia Agricultural Research Institute. To determine the salt stress level, preliminary experiments were conducted to identify the NaCl concentration that inhibited germination and seedling

development by approximately 50%. Germination percentages of the seeds were monitored daily, and by day 5, root-shoot lengths and germination data were evaluated. Based on the results, it was determined that 100 mM NaCl inhibited germination and seedling development by approximately 50% compared to the control, whereas concentrations of 200 mM and above caused 95-100% germination inhibition.

Leonardite-derived humic acid (HA) was used as the humic substance source due to its high humic acid content, low ash content, and natural composition. For this purpose, Agro Elbistan HA product from Tarım 11, sourced from the Maraş-Elbistan region, was used (https://tarim11.com). A preliminary experiment was carried out to determine the most effective HA concentration for promoting plant growth in alfalfa. For this purpose, a stock solution of HA (pH 6-6.5) with a concentration of 5000 mgL^{-1} was first prepared. This stock solution was sterilized in an autoclave and diluted with sterile distilled water to obtain germination media containing HA at concentrations of 0.0, 25, 50, 100, 250, 500, 750, 1000 and 2000 mgL^{-1}. Eight milliliters of these sterile solutions were added to sterile Petri dishes (9 cm) containing two layers of sterile filter paper. Subsequently, 25 seeds were sown into each Petri dish in a sterile cabinet. The Petri dishes were sealed with parafilm. On the 5th day of germination, the root-stem length and germination percentages of the seedlings were recorded. As a result, the concentrations of 500 and 1000 mgL^{-1} HA, which most effectively supported germination and seedling development, were selected for the main experiment. The pot experiments were conducted using one salt concentration (100 mM NaCl) and two humic acid concentrations (500 and 1000 mg L^{-1}) as determined from the preliminary tests.

2.2. Microorganism Isolation

In this study, the microbial material used was an endophytic bacterium, *Bacillus wiedmannii* OG24 (NCBI accession number OQ300504), isolated from a rock lichen (*Rhizoplaca melanophthalma*) collected from Erzurum, Turkey [12]. The bacterial isolation was performed with minor modifications to the method described by Yamamoto et al. (2002) [12, 21]. The endophytic *B. wiedmannii* (Bw) was cultured on Nutrient Agar and incubated for 24 hours at 25 °C. After incubation, bacterial colonies were collected with an inoculation loop and suspended in sterile physiological saline. The suspension was adjusted to an OD600 of 1.00 and applied to the developed plant leaves by spraying until leaf surfaces were visibly wet [12]. The ability of *B. wiedmannii* OG24 (Bw) to degrade HA (use it as a carbon source) was tested, and it was found that 100% degradation occurred after one week of incubation.

2.3. Plant Growth and Incubation Conditions

To ensure plant growth and development, a soil mixture of perlite and peat in a 1:3 (w/w) ratio, previously sterilized by autoclaving, was used in sterilized pots. Prior to sowing, the water-holding capacity of each pot was determined, and salt concentration in the potting medium was maintained constant throughout the experiment. Before sowing, each pot was treated with combinations of distilled water (Control), Salt (100 mM NaCl), HA (500 or 1000 mg L^{-1}), *B. wiedmannii* OG24 (Bw), and all possible combinations to prepare for sowing. The sterilized alfalfa seeds were sown into each pot at a density of 25 seeds. The pots were then transferred to a controlled climate chamber with a 16/8-hour day/night photoperiod and temperature conditions of 25/22 °C. Seedlings (with at least 3-4 leaves) were harvested after a total of 35 days. During harvest, the samples required for further analysis were separated, and the remaining plant material was stored at -80 °C for subsequent analyses.

2.4. Determination of Relative Water Content

After harvesting, the leaves were washed and surface moisture was removed using blotting paper. The fresh weight of the leaves was then measured using a precision balance. Following this, the samples were dried in an oven at 65 °C for 48 hours until a constant weight was achieved, and the dry weight was recorded. Based on these values, the percentage relative water content (RWC%) of the leaves was calculated using the following formula:

RWC (%) = [(Fresh weight - Dry weight) / Fresh weight] x 100

2.5. Determination of Photosynthetic Pigment Content

Chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoid, and total pigment contents were determined according to Witham et al. (1971) [22]. From each sample, 0.25 g of fresh leaf tissue was extracted with 80% acetone. The ground tissue was extracted in 2.5 mL of 80% acetone, filtered, and the volume was adjusted to 5 mL. Absorbance values at 450, 645, and 663 nm were measured, and pigment contents were calculated in mg g^{-1} fresh tissue:

Chl a (mg g^{-1}) = [12.7 (D663) - 2.69 (D645)] x (V/1000 x W)
Chl b (mg g^{-1}) = [22.9 (D645) - 4.68 (D663)] x (V/1000 x W)
Total Chl (mg g^{-1}) = [20.2 (D645) + 8.02 (D663)] x (V/1000 x W)
Total carotenoids (mg g^{-1}) = 4.07 x D450 - (0.0435 x Chl a + 0.367 x Chl b)

Where D is absorbance at the specified wavelength, V is the final volume of 80% acetone, and W is the fresh weight of the tissue extracted.

2.6. Determination of Lipid Peroxidation

Lipid peroxidation (LPO) levels in plant tissues were determined by measuring malondialdehyde (MDA) content. For this, 0.25 g fresh tissue was homogenized in 5% TCA and centrifuged at 12,000 rpm for 15 minutes. To the supernatant, 0.5 mL of 0.5% TBA solution was added, incubated in a boiling water bath for 30 minutes, cooled in an ice bath, and centrifuged at 10,000 rpm for 5 minutes. Absorbance was measured at 532 and 600 nm, and MDA content was calculated [23]:

MDA (μmol mL^{-1}) = [(A532 - A600) - (A450 - A600) x 0.0571] / 157,000 x 10^6

2.7. Determination of H_2O_2 and $O_2^{\cdot-}$ Content

For H_2O_2 quantification, 0.25 g of tissue was homogenized in 2.5 mL cold acetone and centrifuged at 10,000 x g for 10 minutes. Then, 1.5 mL of the supernatant was mixed with 0.15 mL 5% $Ti(SO_4)_2$ and 0.3 mL 19% NH_4OH, centrifuged again, and the precipitate was dissolved in 3 mL 2 M H_2SO_4. Absorbance was measured at 415 nm, and values were calculated in ng g^{-1} using a standard curve [24]. For superoxide anion ($O_2^{\cdot-}$), 0.25 g tissue was homogenized in 2.5 mL 65 mM phosphate buffer (pH 7.8), centrifuged at 5,000 x g for 10 minutes, and 1 mL of the supernatant was mixed with 0.9 mL buffer and 0.1 mL 10 mM hydroxylamine HCl. After 20 minutes at 25 °C, 1 mL of 17 mM aminobenzenesulfonic acid and 1 mL of 17 mM 1-naphthylamine were added and incubated for another 20 minutes. Then, 3 mL of n-butanol was added and absorbance was read at 530 nm. Superoxide levels were determined using a $NaNO_2$ standard curve [23, 25].

2.8. Determination of Antioxidant Enzyme Activities

For antioxidant enzyme activity assays, 0.25 g of plant tissue was ground with liquid nitrogen and homogenized in 0.25 mL of 0.1 M KH_2PO_4 buffer (pH 7.0) containing 1% PVP and 1 mM EDTA. The homogenate was centrifuged at 15,000 x g at 4 °C for 15 minutes. The supernatant was used as the enzyme extract [24, 26].

Superoxide dismutase (SOD; EC 1.15.1.1) activity is based on the inhibition of the reduction of NBT to blue-colored formazan by superoxide radicals in the presence of SOD. The 3 mL reaction mixture prepared for the analysis contained 50 mM KH_2PO_4 (pH 7.8), 13 mM methionine, 63 μM NBT, 13 μM riboflavin, and 0.1 mM EDTA. 2.58 mL of the riboflavin-free mixture was taken into a spectrophotometer cuvette, and 30 μL of enzyme extract was added. The reaction was carried out in front of a white light source for 15 minutes after the addition of 390 μL of 13 μM riboflavin, and absorbance values were taken at 560 nm at the end of this period. The amount of enzyme that provided 50% inhibition of NBT reduction compared to the enzyme-free control group was defined as 1 enzyme unit. The results are presented as EU mg $protein^{-1}$ [26, 27]. Catalase (CAT; EC 1.11.1.6) enzyme activity was determined by measuring the decrease in absorbance at 240 nm during the breakdown of H_2O_2 into water and oxygen. For measurement, a reaction mixture containing H_2O_2 solution and enzyme extract was prepared, and the change in absorbance was monitored at 240 nm for 3 minutes in a spectrophotometer. The values calculated from the minute decrease in absorbance were converted to H_2O_2 amount using the molar absorptivity coefficient (ε = 39.4 mM^{-1} cm^{-1}), and the results were expressed in EU mg $protein^{-1}$ [26]. Guaiacol peroxidase activity (GPX; EC 1.11.1.7) was measured based on the increase in absorbance at 470 nm of the colored compound formed as a result of the reaction of guaiacol with H_2O_2. For the analysis, 10 μL of enzyme extract was added to 3 mL of substrate solution containing 0.1 M KH_2PO_4 (pH 5.5) and 5 mM guaiacol. The increase in absorbance was measured at 470 nm every minute for a total of 5 minutes. The amount of enzyme that caused an increase of 0.01 absorbance per minute was accepted as 1 enzyme unit (EU), and the results were presented in EU mg $protein^{-1}$ [26, 27]. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined based on the decrease in absorbance observed at 290 nm during the reaction of ascorbic acid (ASA) with H_2O_2 [28]. The reaction mixture was prepared to contain 50 mM potassium phosphate buffer (pH 7.0), 250 μM ASA, 5 mM H_2O_2, and 100 μL of enzyme extract. The obtained absorbance changes were measured at 290 nm, and the enzyme activity was calculated using the epsilon coefficient (2.8 mM^{-1} cm^{-1}), and the results were expressed as EU mg $protein^{-1}$. Glutathione reductase (GR; EC 1.6.4.2) activity was determined by a spectrophotometric method based on the oxidation of NADPH [29]. The reaction mixture was prepared by dissolving 200 μL of 0.5 mM EDTA, 250 μl of GSSG, and 500 μL of NADPH solutions in 2.95 mL of 50 mM Tris-HCl buffer (pH 7.8). A 50 μL of enzyme extract was added to this mixture. The obtained absorbance changes were measured at 340 nm, and the enzyme activity was calculated using the epsilon coefficient of NADPH (ε = 6.22 mM^{-1} cm^{-1}), and the results were expressed as EU mg $protein^{-1}$.

2.9. Statistical Analysis

Data were presented as mean values from three independent biological replicates (n=3), each with two technical replicates (total of six measurements). Statistical analyses were performed using SPSS 21.0 software. One-Way ANOVA was used to determine significant differences among groups, and Duncan's Multiple Range Test was applied for post-hoc analysis. Differences were considered statistically significant at $p<0.05$.

3. RESULTS

Our preliminary experiments indicated that 100 mM NaCl imposed a moderate level of salt stress on the alfalfa by reducing both seed germination and seedling growth by approximately 50%. The salt dozes of 200 mM NaCl and above caused 95-100% germination inhibition. Based on this, 100 mM NaCl was chosen as the stress-inducing concentration for the main pot experiments. Additionally, HA did not significantly affect seedling development at doses below 500 mg/L compared to the control ($p<0.05$), but the doses above 1000 mg/L significantly inhibited seedling growth. During the same study, the bacterium was applied to a group of the developing alfalfa seedlings, and it was determined that the bacteria did not exhibit any pathogenicity in the developing alfalfa seedlings (As shown in Figure 1, this finding has also been observed in pot experiments.).

With regard to relative water content (RWC) of leaves, neither HA nor the bacterial isolate (*Bacillus wiedmannii*) treatments alone resulted in significant ($p<0.05$) differences compared to the control group (90.54%)

(Figure 1). In contrast, the exposure to salt stress alone led to a substantial decrease in RWC, reducing it to 82.5%.

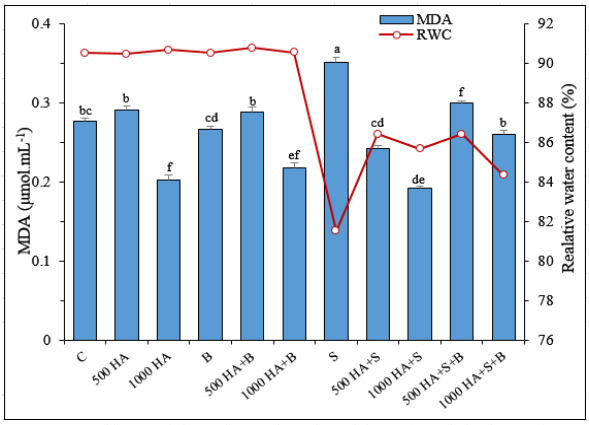

Figure 1. Effects of the salt (S), humic acid (HA) and the bacterium (B) treatments on MDA and RWC contents. Differences between groups with the same letters are insignificant according to Duncan's Multiple Comparison test ($p<0.05$).

However, the combined application of HA and *B. wiedmannii* (Bw) under the saline conditions mitigated this adverse effect. For example, in the treatment group receiving 1000 mg L^{-1} HA and Bw under salt stress, RWC improved to 85.7%, indicating a partial restoration of water status in the leaves.

3.1. Effect of Treatments on ROS and MDA Content

The salt stress significantly ($p<0.05$) increased both H_2O_2 and $O_2^{\cdot-}$ levels in *M. sativa* seedlings compared to the control group (Figure 2). The highest levels of H_2O_2 (6 µM) and $O_2^{\cdot-}$ (10.5 nmol mg $protein^{-1}$) were observed in the group exposed to salt stress alone. While the application of HA (500 and 1000 mg L^{-1}) alone led to a slight increase in H_2O_2 and $O_2^{\cdot-}$ levels, the Bw treatment did not cause a marked change in these parameters and tended to reduce superoxide accumulation in particular. When HA and Bw were applied together, the partial reductions were observed in both ROS levels. Under the salt stress, individual applications of HA and Bw reduced MDA level, but the most notable reductions were recorded in the groups where HA and Bw were applied in combination. Especially 1000 mg L^{-1} HA+Bw+Salt group significantly reduced H_2O_2 and $O_2^{\cdot-}$ levels compared to the salt-stressed group, making it the most effective combination in suppressing oxidative stress. These results indicate that the combined application of HA and *B. wiedmannii* provides a synergistic protective effect against salt stress. Compared to the control group, the HA treatments significantly ($p<0.05$) reduced MDA levels, lowering them to 0.23 and 0.30 µmol mL^{-1}, respectively (Figure 1). When applied alone, the bacterium decreased MDA levels to 0.27 µmol mL^{-1}, showing a moderate reduction compared to the control. However, the HA+Bw combinations did not show a significant effect relative to the control. Under stress conditions induced by the salt treatment, MDA levels rose to 0.36 µmol mL^{-1}, representing the highest value among all groups ($p<0.05$). In the HA+Salt combinations, MDA levels were reduced again to 0.23 and 0.27 µmol mL^{-1}, indicating that high-dose HA effectively mitigated oxidative stress even under salt stress conditions. In combinations involving both salt and HA with Bw, MDA levels were measured at 0.30 µmol mL^{-1} in the 500 HA+Salt+Bw group and 0.27 µmol mL^{-1} in the 1000 HA+Salt+Bw group.

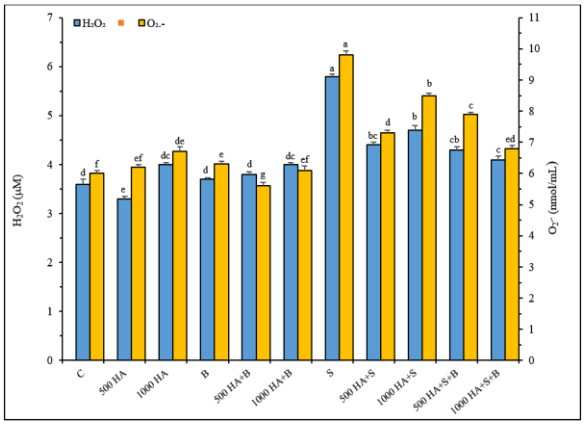

Figure 2. Effects of the salt (S), humic acid (HA) and the bacterium (B) treatments on H_2O_2 and $O_2^{\cdot-}$ MDA and RWC contents. Differences between groups with the same letters are insignificant according to Duncan's Multiple Comparison test ($p<0.05$).

3.2. Effects of Treatments on Antioxidant Enzyme Activities

SOD activity was approximately 1.8 EU mg $protein^{-1}$ in the control group (Figure 3). The HA (500 mg L^{-1}) treatment slightly increased SOD activity, helping to maintain the enzyme activity, while 1000 HA treatment showed a slightly lower activity. The bacterial treatment resulted in a similar level of activity. Combinations of the HA and the bacterium led to the highest levels of SOD activity, reaching around 2.0 EU mg $protein^{-1}$. Under the salt stress, SOD activity significantly ($p<0.05$) decreased to about 1.4 EU mg $protein^{-1}$. The Salt+HA treatments partially restored SOD activity, however, the most notable increases were observed in the 500 HA+Salt+Bw and 1000 HA+Salt+Bw groups, which elevated SOD activity back to approximately 1.8-1.9 EU mg $protein^{-1}$. On the other hand, CAT activity, compared to the control group (1.3 EU mg $protein^{-1}$), was not significantly ($p<0.05$) altered by HA or Bw treatments alone (Figure 3). Similarly, HA+Bw combinations did not cause a meaningful change in CAT activity. The salt treatment significantly reduced CAT activity to about 1.0 EU mg $protein^{-1}$ compared to the control. Salt+HA treatments provided slight increases in CAT activity, with a significant improvement particularly observed in the 1000 HA+Salt group. The most remarkable increases were determined in the 500 HA+Salt+Bw and 1000 HA+Salt+Bw groups.

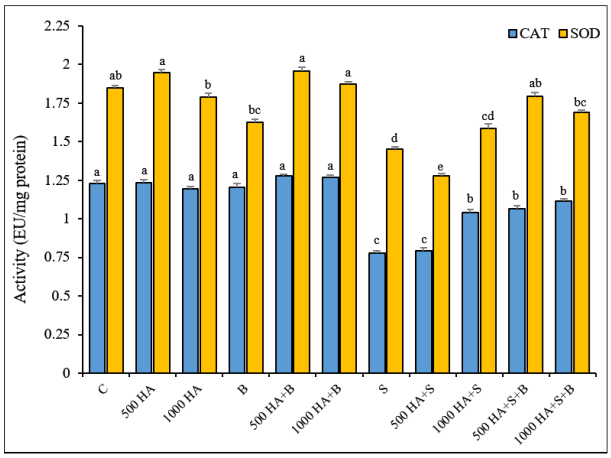

Figure 3. Effects of the salt (S), humic acid (HA) and the bacterium (B) treatments on CAT and SOD activity. Differences between groups with the same letters are insignificant according to Duncan's Multiple Comparison test ($p<0.05$).

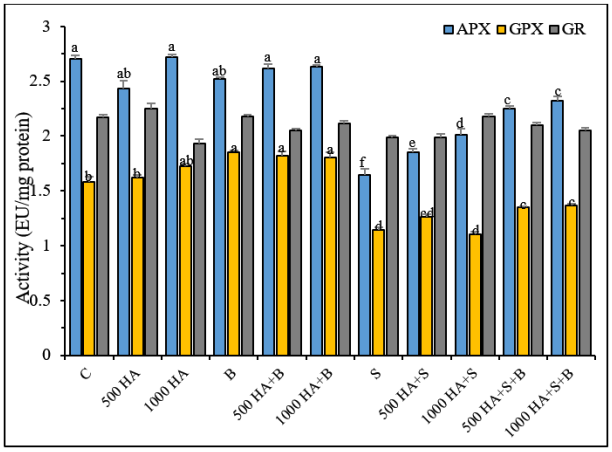

Figure 4. Effects of the salt (S), humic acid (HA) and the bacterium (B) treatments on APX, GPX, and GR activity. Differences between groups with the same letters are insignificant according to Duncan's Multiple Comparison test ($p<0.05$).

APX activity was not significantly affected by HA and Bw applications compared to the control (Figure 4). However, the salt stress significantly reduced APX activity in the seedlings, with activity decreasing to approximately 1.6 EU mg protein^{-1}. When HA+Salt treatment was applied together, APX activity significantly ($p<0.05$) increased compared to the salt alone. More pronounced increases in APX activity were observed in the groups where both HA and Bw were applied under salt stress. As for GPX activity, HA and Bw treatments did not result in a significant increase compared to the control and remained at similar levels (Figure 4). The salt application caused a substantial decrease in GPX activity, reducing it to 1.1 EU mg protein^{-1}. In the groups treated with salt in combination with 500 or 1000 mg L^{-1} HA, slight but significant increases in GPX activity were observed. The most notable increases occurred in the groups treated with both HA and Bw under the salt stress, where GPX activity reached approximately 1.6-1.7 EU mg protein^{-1}. In terms of GR activity, HA and Bw treatments alone caused slight, non-significant increases compared to the control (Figure 4). In the salt-stressed group, GR activity was significantly reduced, dropping to around 1.5 EU mg protein^{-1}. In groups where HA and salt were applied together, significant increases in GR activity were observed, with a more noticeable rise particularly in the 1000 HA+Salt group. The most remarkable recovery was detected in the groups where both HA and Bw were applied under salt stress, with GR activity reaching up to 2.0 EU mg protein^{-1}. These results demonstrate that the combined application of HA and *B. wiedmannii* is effective in restoring the antioxidant enzyme activities to normal levels under the salt stress conditions.

3.3. Effects of Treatments on Photosynthetic Pigment Content

When salt stress was applied alone, all Chl parameters declined significantly ($p<0.05$), and the proportional decrease in Chl a was greater than that of Chl b (Figure 5). HA treatments, especially at the high dose (1000 mg L^{-1}), induced significant increases in Chl a, Chl b, and total Chl relative to the control. Notably, the enhancing effect of 1000 mg L^{-1} HA was more pronounced on Chl a than on Chl b. The Bw inoculation alone also elevated Chl content, with the increase in Chl a slightly exceeding that in Chl b, although this rise was less marked than with HA treatments. Combinations of the salt stress with HA and Bw treatments partially alleviated the adverse effects of the salinity. In particular, the 1000 mg L^{-1} HA+Salt+Bw treatment produced a significant ($p<0.05$) recovery in Chl levels compared to salt alone. Across these combined treatments, improvements in Chl a were generally more pronounced than those in Chl b.

The application of salt alone reduced the carotenoid content compared to the control. However, individual applications of 1000 mg L^{-1} humic acid (HA) and bacteria significantly increased carotenoid levels (Figure 5). Notably, the highest carotenoid content (approximately 0.26) was observed in the group treated solely with bacteria. The 500 mg L^{-1} HA treatment and the combination applications (500 HA+Bw, and 1000 HA+Bw) also showed an increase compared to the control, although the increase in the 1000 HA+Bw group was not considered statistically significant. When the effects of HA+Bw applications under salt stress were examined, it was found that in the 500 HA+Salt and 1000 HA+Salt groups, the negative impact of salt was partially mitigated. However, carotenoid levels in these groups still remained below the control level. In the groups where bacterial treatment was combined with salt (Salt+Bw and 1000 HA+Salt+Bw), carotenoid levels were observed to be higher than in the group treated with salt alone.

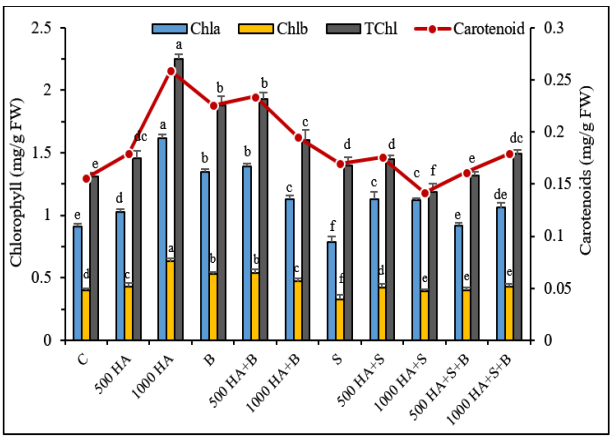

Figure 5. Effects of the salt (S), humic acid (HA) and the bacterium (B) treatments on photosynthetic pigment content. Differences between groups with the same letters are insignificant according to Duncan's Multiple Comparison test ($p<0.05$). FW: Fresh weight.

4. DISCUSSION

In this study, humic acid (HA) derived from leonardite, produced in the Maraş-Elbistan region (Türkiye) was preferred due to its content and natural origin (https://tarim11.com). This HA is a domestically produced humic acid obtained from natural leonardite with a pH range of 2-4, containing 12% total organic matter, 12% total humic acids, 2.5% water-soluble potassium oxide (K_2O), and a final pH range of 7-9 [30]. Leonardite-based HA exerts a strong effect on plants due to its high humic content. Additionally, being a natural and organic source allows for environmentally friendly use. Compared to other HA sources, leonardite has a lower ash content, which means it does not increase soil salinity or inhibit microbial activity [7,31,41]. In contrast, HA derived from lignite and coal generally have lower organic matter content and limited solubility, reducing their effectiveness on plant growth [32, 33]. Synthetic HAs, on the other hand, are not obtained from natural sources, making them unsuitable for use in organic farming and potentially more harmful to the environment [31]. For these reasons, leonardite-based HA offers a more effective and sustainable solution compared to other sources.

Relative water content (RWC) of leaves reveals the impact of stress on the plant's water balance. In the control group, RWC was measured at 90.54%, whereas in the plants exposed only to the salt stress, this value decreased to 82.5% (Figure 1). This decline aligns with literature indicating that salt reduces osmotic potential, thereby limiting water uptake and increasing water loss in plants [34]. However, under the salt stress, significant improvements in RWC values were observed with the application of HA and *B. wiedmannii* (Bw) Notably, in the combination of 1000 mg L^{-1} HA+Bw+Salt, the RWC value increased to 85.7%, indicating the potential of these treatments to enhance water retention capacity. This ameliorative effect is likely due to HA promoting root development, thereby facilitating water uptake, and the bacterial isolate (*B. wiedmannii*) enhancing the plant's response to salt stress [35].

Salt stress is a significant abiotic factor that induces oxidative damage in plants by increasing the accumulation of ROS such as H_2O_2 and $O_2^{\cdot-}$ [5, 34]. In this study, compared to the control, the individual applications of HA, bacteria, or HA+Bw did not have a strong or statistically significant effect on the ROS content in the seedlings leaves (Figure 2). In contrast, salt stress significantly increased H_2O_2 and $O_2^{\cdot-}$ levels compared to the control. High salt concentrations can trigger ROS production by causing ionic imbalances in cells, denaturation of proteins, and reduced photosynthetic activity [5, 34]. However, a synergistic effect in suppressing oxidative stress was observed in the groups where HA and the bacteria were applied together. Specifically, in the 1000 mg L^{-1} HA+Bw+Salt, H_2O_2 and $O_2^{\cdot-}$ levels were significantly lower than in the salt-only group, indicating that this combination is much more effective in protecting plants against salt stress than the individual treatments (Figure 2). This synergistic effect may be attributed to HA improving the plant's physiological state, thereby enhancing the effectiveness of the bacterium's stress-mitigating mechanisms, or to the bacterium facilitating the uptake and utilization of HA's bioactive components by the plant. When examining MDA levels, an indicator of LPO (Figure 1), it was found that both HA and the bacterial treatments alone significantly reduced MDA content compared to the control, supporting their individual potential to mitigate oxidative damage. Salt treatment significantly increased MDA levels, aligning with existing literature indicating membrane damage due to salt stress [5, 32]. Reductions in MDA levels were also observed in HA+Salt and HA+Salt+Bw treatments. Notably, the low MDA level in the 1000 mg L^{-1} HA+Salt+Bw provides further evidence of the synergistic effect of HA and bacteria in reducing salt-induced membrane damage. These findings demonstrate that the combined application of HA and *B. wiedmannii* significantly alleviates oxidative stress and suppresses lipid peroxidation in *M. sativa* under salt stress through a synergistic mechanism.

SOD isoforms the first line of defense in plants against free radicals by converting superoxide anions into H_2O_2 [5]. Salt stress typically elevates intracellular ROS levels and acts as a trigger to stimulate SOD activity. For example, Younesi and Moradi (2014) reported that 200 mM NaCl significantly increased SOD activity in alfalfa seedlings [37]. In contrast, in our study, under 100 mM salt stress, SOD activity decreased compared to control levels (Figure 3). This reduction may be associated with the salt tolerance level of the cultivar used. Indeed, the alfalfa variety (Konya) used in this study exhibited 50% growth inhibition at 100 mM salt and failed to germinate or grow at 200 mM. According to the literature, salt-tolerant cultivars tend to have higher SOD activity, enabling them to better cope with salt-induced stress. For instance, salt-tolerant rice varieties have shown elevated expression of SOD genes and higher SOD activity compared to salt-sensitive varieties [5]. However, this decline in SOD activity was partially mitigated by the application of HA or Bw, and the combination of HA+Bw even restored SOD activity to near control levels. Similarly, HA treatments have been shown to enhance salt

tolerance in wheat by boosting antioxidant system activity, including SOD [8]. In addition, inoculation with Bacillus PGPR strains has been reported to increase SOD activity by 25-50% in salt-stressed rice plants [38]. In our findings as well, inoculation with *B. wiedmannii* led to a noticeable increase in SOD activity, which returned to near-control levels when applied in combination with HA. Similarly, CAT activity significantly decreased under 100 mM NaCl treatment, but substantial increases in CAT activity were observed with the combined application of 500 and 1000 mg L^{-1} HA and the bacterium (Figure 3). In a similar study, applications of *B. subtilis* and *B. aryabhattai* significantly enhanced CAT activity in salt-stressed rice and tomato plants [39, 40]. Likewise, HA has been reported to mitigate oxidative damage in plants and strengthen enzymatic defenses [9]. Additionally, Bacillus consortia with catalase-producing ability have been shown to increase CAT activity by more than 50% [40].

APX is one of the key enzymes responsible for detoxifying H_2O_2 within the ascorbate (AsA)-glutathione (GSH) cycle. Under saline conditions, APX activity typically increases; for instance, Younesi and Moradi (2014) reported that 200 mM NaCl more than doubled APX activity in alfalfa [37]. High APX expression and activity in salt-tolerant genotypes have also been reported in the literature [5]. However, in our study, APX activity decreased under the salt stress in the alfalfa (Figure 4). This decline may be related to the salt sensitivity of the specific cultivar. As noted earlier, the Konya variety of alfalfa showed 50% growth inhibition at 100 mM salt and failed to germinate at 200 mM. On the other hand, while individual applications of HA or bacteria led to a slight recovery, the HA+Bw combination restored APX activity to near-control levels. Some studies have suggested that HA can enhance APX activity. For example, HA application increased APX activity in salt-stressed sorghum plants [38]. Similarly, Bacillus PGPR inoculation has been shown to increase APX activity in rice by 45-58% [39]. Furthermore, co-application of PGR and HA generally enhances antioxidant enzyme activities and reduces peroxidation levels, which indicate cellular damage [43]. These findings demonstrate that the combination of HA and *B. wiedmannii* helped restore APX activity to near baseline levels.

GPX is a type of peroxidase that uses phenolic substrates to break down H_2O_2 and protects cell membranes from oxidative damage [5]. In many plant species, increased peroxidase activities have been observed under salt stress conditions [44]. This suggests that plants activate the GPX pathway to mitigate salt-induced ROS accumulation. In our findings, however, GPX activity was reduced under the salt stress (Figure 4). A study on ryegrass reported that antioxidant enzyme activities, including GPX, initially increased under salt stress but began to decline after the 12th day of exposure. This indicates that antioxidant enzymes may be activated during the early phase of stress as a defense response but can become suppressed as stress persists [45]. The alfalfa seedlings in our study were subjected to 35 days of salt stress, the observed decline in GPX activity is consistent with this pattern. On the other hand, the decline in GPX activity due to the salt stress was partially recovered with the application of either HA or bacteria, and near-control levels were achieved with the combined HA+Bw treatment. Literature supports the role of HA in enhancing GPX activity and protecting plants from oxidative stress [8, 39]. Similarly, Bacillus inoculation has been shown to positively influence GPX activity in crops like wheat and maize [8]. Therefore, these findings indicate that the combined application of HA+*B. wiedmannii* helps restore GPX activity in salt-stressed alfalfa, thereby supporting cellular ROS detoxification mechanisms.

GR is a crucial component of the AsA-GSH cycle in plants. This enzyme regenerates ascorbate (AsA) from dehydroascorbate via DHAR and also converts oxidized glutathione (GSSG) back to its reduced form (GSH), thereby maintaining redox homeostasis [5]. Under salt stress, changes in GR activity are often reported to be more limited. For instance, in alfalfa, salt stress was found to increase SOD and APX activities, while no significant change in GR activity was observed [37]. This suggests that GR tends to remain more stable in order to preserve the integrity of the AsA-GSH cycle. In the study, however, GR activity decreased under salt stress compared to the control, and this reduction was only partially alleviated by either HA or bacterial treatments alone (Figure 4). This response may be closely related to the salt tolerance level of the alfalfa variety used and the duration of salt exposure. Notably, the combined application of HA+Bw restored GR activity to levels close to that of the control group. Overall, it is known that HA and PGPR applications enhance the AsA-GSH cycle by activating GR and other associated enzymes, thereby improving H_2O_2 detoxification [43]. Our findings are consistent with this mechanism, as the HA+*B. wiedmannii* treatment effectively restored GR activity, supporting the antioxidant defense system in the salt-stressed alfalfa.

Salt stress is well known to impair photosynthetic pigment synthesis and accelerate pigment degradation in plants, often leading to lower Chl a, Chl b, and total Chl contents [46]. In this study, 100 mM NaCl alone elicited a marked decline in Chl a and Chl b compared to control plants, with Chl a exhibiting a proportionally larger reduction than Chl b (Figure 5). These results are consistent with previous findings in alfalfa, where salinity reduced Chl content and impaired photosystem II (PSII) efficiency, but did not significantly affect activity at lower salinity levels [46]. The HA treatments, especially at 1000 mg L^{-1}, significantly increased Chl a, Chl b, and total Chl relative to control plants, with a more pronounced effect on Chl a than Chl b. In addition, Sofi et al. (2018) demonstrated that HA improved Chl a, Chl b, and total Chl in alfalfa seedlings exposed to various salinity levels, particularly under 120 mM NaCl [47]. Similarly, studies in maize and cucumber showed that HA application ameliorated salinity effects by elevating pigment contents, and maintaining RWC, and enhancing antioxidant enzyme activities. In this study, *B. wiedmannii* inoculation alone slightly increased Chl a and Chl b (with a slightly higher relative rise in Chl a) compared to controls, although to a lesser extent than HA treatments. These increments align with evidence that PGPR can enhance pigment synthesis

by improving water relations and osmolyte accumulation [48, 49]. Specifically, in alfalfa, inoculation with Bacillus strains under saline conditions prevented Chl loss by lowering chlorophyllase activity and preserving PSII functionality [48, 49]. When HA and Bw were combined under salt stress (100 mM NaCl), partial recovery of Chl contents was evident. The 1000 mg L^{-1} HA+Salt+Bw treatment yielded a significant restoration of Chl a and Chl b relative to the salt alone, with Chl a improvements more pronounced.

Carotenoids play essential roles in photoprotection by scavenging ROS and dissipating excess excitation energy under abiotic stress. It is well documented that salinity decreases carotenoid content, compounding oxidative damage [50, 51]. In this study, the salt alone reduced carotenoid levels below those of controls. Conversely, individual HA (1000 mg L^{-1}) and the bacterial treatments significantly increased carotenoid content, with *B. wiedmannii* alone achieving the highest carotenoid concentration (Figure 5). Similarly, Bacillus-mediated carotenoid enhancement has been observed in rice and tomato under salt stress, where PGPR-induced antioxidant enzyme activation coupled with elevated carotenoid synthesis mitigated oxidative damage [49, 50]. Furthermore, in Salt+Bw and 1000 HA+Salt+Bw, carotenoid concentrations exceeded those in the salt alone, underscoring the superior efficacy of Bw (with or without HA) in bolstering carotenoid defenses under salinity. This suggests that the bacillus-induced modulation of carotenoid biosynthesis may be more potent than HA alone for restoring antioxidant capacity in alfalfa under salt stress [49, 50]. Taken together, this data demonstrates that HA (1000 mg L^{-1}) and *B. wiedmannii* can mitigate salt-induced declines in photosynthetic pigments in alfalfa, with combined applications offering superior recovery.

5. CONCLUSION

This study demonstrated that the application of HA derived from the leonardite and endophytic *Bacillus wiedmannii* OG24 from the lichen reduced the adverse effects of the salt stress (100 mM NaCl) on the *Medicago sativa* seedlings. The combination of HA and the bacterial treatments significantly increased RWC, reduced ROS level and LPO, and restored the activities of key antioxidant enzymes (SOD, CAT, APX, GPX, and GR) that were suppressed under the salt stress. Additionally, both HA and *B. wiedmannii* helped restore the photosynthetic pigment levels, with the combined treatment showing the most pronounced improvements. Overall, these improvements can be attributed to the restoration of mechanisms such as ion balance, osmotic regulation, antioxidant defense, and water-holding capacity in the plant. For example, HA can increase the K^+/Na^+ ratio in the plant by enhancing K^+ uptake and limiting Na^+ accumulation, and it also reduces LPO level and ion leakage by increasing antioxidant enzyme activities [9]. In this case, oxidative stress is suppressed while photosynthesis and water uptake are supported. With the supportive environment effect in the soil, root development is stimulated, and the RWC in the plant increases, thereby enhancing water-holding capacity [41]. We believe that the *B. wiedmannii* OG24 isolate supports plant growth through various PGPR mechanisms. Such strains exhibit characteristics such as high indole-3-acetic acid (IAA) production, phosphate solubility, and nitrogen fixation [17, 43]. Additionally, exopolysaccharide secretion facilitates water retention and bacterial colonization in the rhizosphere [52]. ACC deaminase activities also promote growth by reducing stress ethylene in plants [24]. HA also enriches the root environment with organic acids and nutrients, enhancing the attachment and efficacy of such bacterial isolates to roots [43]. According to our findings, such a synergistic interaction could explain the increased stress response observed in the *M. sativa* under salt stress. In conclusion, the combined application of HA derived from leonardite and *B. wiedmannii* demonstrates a promising, environmentally friendly strategy to enhance salt stress resistance in alfalfa by strengthening physiological, biochemical, and antioxidant defense responses.

Author Contributions

Ö.A. was responsible for the study design, conducting the experiments, literature review, and manuscript writing. A.M. contributed to the experimental work and literature review. Ö.G. was involved in conducting the experiments and performing statistical analyses.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding this study.

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