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Article

The Effects of *Halomonas* sp. and *Azotobacter* sp. on ameliorating the adverse effect of salinity in purple basil (*Ocimum basilicum* L.)

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Abstract: The role of plant growth-promoting rhizobacteria (PGPR) on enhancing tolerance of plants to abiotic stresses is well reported, but the effects of RGPRs on plants under salinity stress are not widely studied in the literature. Our study aimed to investigate the effect of *Halomonas* sp. and *Azotobacter* sp. on antioxidant activity, secondary metabolites, and biochemicals changes of purple basil under salinity stress conditions. The applied salt concentrations in this study were 50, 100, and 150 mM sodium chloride (NaCl). *Salinity stress had* a negative *effect* on plant growth *parameters*. Moreover, a reduction in some of the osmolytes and oxidative stress markers was observed. Inoculated plants ameliorated the oxidative damage by reducing the hydrogen peroxide (H_2O_2) contents and by increasing osmolytes (proline, total proteins, and soluble sugars), antioxidant enzymes activities (catalase, ascorbate peroxidase) and secondary metabolites (flavonoids). Overall, among treatments, plants inoculated with *Azotobacter* showed a better impact on physiological attributes to alleviate the adverse effects of 150 mM NaCl salinity stress on basil growth.

Keywords: catalase; flavonoids; hydrogen peroxide; proline, soluble sugars

1. Introduction

Basil (*Ocimum basilicum* L.) is one of the essential vegetables and medicinal plants belong to the family Lamiaceae, with a great diversity in morphology, biology, essential oils content, and chemical compounds [1]. This plant is traditionally used for the treatment of intestinal problems, respiratory and kidney disease. This plant is economically very important because its essential oil is being used in perfumes, cosmetics and cleaning products, and has a local anaesthetic and antiseptic effects [2].

Abiotic stresses such as salinity and drought are important factors in reducing plant growth. It affects the global food production directly and indirectly [3]. In the natural ecosystems of arid and semi-arid areas, saline soil creation can decrease the soil productivity. In these areas, irrigation leads to secondary salinization and disturbs the crops growth [4]. The soil salinity might reduce the productivity of most vegetables, as the low salinity threshold (ECt) of the majority of vegetable [5]. Understanding the salinity tolerance in vegetables is crucial to increase their productivity and can help us increase the profitability of crops irrigated with saline water. Ionic imbalance, toxicity, and decreasing water potential of the plants are the most important adverse effects of the salinity stress on the plants [6]. High level of sodium (Na^+) ion in plant tissues prevents photosynthesis and increases the reactive oxygen species (ROS). It can induce a negative effect on the plant physiology by the protein breakdown, DNA and RNA mutation as well as with the membrane destruction. Therefore, maintaining the osmotic balance and ionic stability in plants is essential for overcoming the adverse effects caused by the salinity stress [7].

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Irrigation water salinity mainly exacerbates the soil alkalization and salinization. It also affects the available soil nitrification and denitrification, enzymes, nitrogen and microorganisms. This will increase the soil N2O emission, an important greenhouse gas. The photochemical reactions of N2O can affect the destruction of the ozone layer and increase the global warming [8].

There are several ways to improve the production of crops under salinity stress conditions, including the proper management of agricultural fields, the use of transgenic plants, and plant growth-promoting bacteria (PGPR) [9]. It should be noted that the use of transgenic plants is highly controversial and requires careful evaluation of its possible risks before generalization [10]. Microbial sources are safer and more environmentally friendly alternatives that can effectively increase agricultural products [11].

Researchers steadily are working on abiotic stress through biotechnology and using advanced techniques to improve the plant's tolerance to unfavorable environmental conditions. For example, PGPRs are widely used in sustainable agriculture systems. However, in the last two decades, many scientists have inoculated the plant roots system with PGPR and improved the plant's tolerance to abiotic and biotic stresses. Rhizosphere colonization by PGPR causes changes in the physical and chemical properties of the plants, increasing their defense and tolerance to stresses [12]. Plant inoculation with PGPRs can create Induced systemic resistance (ISR) in the plant [13]. ISR is the process which PGPRs reduce the deleterious effects of plant pathogens by activating a resistance mechanism. ISR targets no specific pathogens but rather prepares the plant against a variety of pathogens and it is worthy to say that ISR is not only expressed at the site of induction [14]. It has been reported that PGPRs play an important role in improving water-plant relations, ionic homeostasis, and photosynthetic efficiency under salinity stress [15]. Furthermore, pieces of evidence suggest that PGPRs help the water and nutrient uptake, plant hormone status, signal transmission proteins, antioxidant enzymes activities, and carbohydrate metabolism in plants by ISR. For example in a study it is reported that inoculation of the pepper plant with *Azospirillum brasilense* and *Pantoea dispersa* under salinity stress increased stomatal conductance and photosynthesis but did not affect chlorophyll content and photochemical efficiency of photosystem II [16]. Inoculation of pea plant with *Halomonas variabilis* HT1 and *Planococcus rifietoensis* RT4 has shown to increase the growth of the plant and improve the salinity tolerance of the plant up to 200 mM NaCl [17].

Concerning the crucial roles of the PGPRs on growth and increasing the tolerance to biotic and abiotic stresses in plants, the objective of this study is to evaluate the effects of *Halomonas* sp. and *Azotobacter* sp. on physiological and biochemical responses, antioxidant activity, and secondary metabolites of purple basil under salinity stress. To do so, after determining the salinity stress response of purple basil, first, we conduct an experiment to understand if *Azotobacter* sp., *Halomonas* sp. (and mixture of them) will be effective on the indicated factors under normal condition. Moreover, if they are effective on non-saline condition, they will be tested in purple basil under saline condition.

2. Materials and Methods

2.1. Plant Material and Treatments

This research was conducted in the Department of Horticultural Science laboratory, Shiraz University, Shiraz, Iran. In this study, purple basil seeds were purchased from Fardin Kesht Company. The *Halomonas* sp. and *Azotobacter* sp. bacteria were grown on nutrient broth medium at 28 ± 2 °C for 24 h on a shaker (150 rpm). For *Halomonas* 7% NaCl added to nutrient broth medium. A good bacterial density was measured at 600 nm. For inoculation of plants with *Halomonas* sp. and *Azotobacter* sp, inoculum having 10^5–10^6 CFU/ml was prepared by centrifuging freshly grown bacterial culture at 10,000 g for 5 min. The seeds were planted in plastic pots (0.5 L volume) filled with perlite. Four seeds were planted in each pot and irrigated with distilled water for two weeks. Pots were kept under controlled conditions inside the germinator (relative humidity 75%, night temperature 19 °C, day temperature 25 °C, 3000 lux light for 16 hours).

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This study was performed as a factorial experiment in a complete randomized design with four replications. Treatments were at four levels of control, *Halomonas* sp. (H), *Azotobacter* sp. (A) and *Halomonas* sp. + *Azotobacter* sp. (A+H), and salinity treatments were at four levels (0 (S0), 50 (S1), 100 (S2), 150 (S3) mM NaCl).

Seedlings were fertigated after emerging of cotyledon leaves with 1/4 Hoagland solution, and after appearing first true leaves by half Hoagland solution, and then fed with a full Hoagland solution till the end of the experimental period. Salinity treatments were applied after reaching plants to the desired size (6-8 leaves). Salinity stress gradually applied and performed for three weeks based on Hoagland and Arnon nutrient solution concentration [18].

2.2. Growth Parameters

At the end of the experiment, the plants were removed from the pot, and fresh weight was measured. Then, samples were placed in the oven at 80 °C for 48 h, and dry weight was calculated. The leaf area index (LAI) was measured by Fiji software [19].

2.3. Biochemical Attributes

2.3.1. Chlorophylls (a, b, total) and Carotenoids Contents

The chlorophyll content (a, b, total), and carotenoids of the leaves were measured using dimethyl sulfoxide (DMSO). Briefly, 0.1 g of leaves were placed inside the test tube, then 7 ml of DMSO was added and placed in an incubator at 65 °C for 30 min. The supernatant was removed into the Erlenmeyer flasks. The extract volume was increased to 10 ml by adding DMSO. Finally, absorbance is measured at 645, 663, and 470 nm using a spectrophotometer (Epoch, Biotech, USA). DMSO was used as blank. The chlorophylls (a, b, total) and carotenoids were reported as mg g^{-1} of fresh weight (mg g^{-1}FW) and calculated with the following formulas [19,20]:

$$\text{Chlorophyll a (mg/g.FW)} = \frac{12.7(A663)-2.69(A645)\times \text{Volume}}{Wt}$$

$$\text{Chlorophyll b (mg/g.FW)} = \frac{22.9(A645)-4.68\,(A663)\times \text{Volume}}{Wt}$$

$$\text{Chlorophyll total (mg/g.FW)} = \frac{20.2\,(A645)+8.02(A663)\times \text{Volume}}{Wt}$$

$$\text{Carotenoids (mg/g.FW)} = \frac{1000\,(A470)-1.82C_a-85.02C_b}{198}$$

Where Wt is the fresh weight of the sample, C_a is chlorophyll a content, C_b is the chlorophyll b content, Ax is the absorbance at λwavelength (nm).

2.3.2. Soluble Carbohydrates Content

For measurement of soluble carbohydrates content, 0.1 g of powdered dried leaf sample was put into the Falcon tube. Then, 10 ml of 80% ethanol was added and centrifuged at 5,000 rpm for 10 min. The supernatant was transferred into Falcon, and 10 ml of 80% ethanol was added to the previous precipitate and centrifuged at 5000 rpm for 10 min, and the supernatant was added to the previous Falcon. Then, 5% phenol was made, and 25 µl of the solution was added into the microplate. After that, 25 µl of 5% phenol solution was added to each microplate cell containing the extract. Immediately, 125 µl of pure sulfuric acid was added to each microplate cell. Samples were incubated at 25-30 °C for 30 min. Then, the samples' absorbance measured at 490 nm by a microplate spectrophotometer (Epoch, Biotech, USA). Different concentrations of glucose (mg g^{-1} DW) were used to prepare the standard curve [21].

2.3.3. Starch Content

Briefly, 200 µl of cold distilled water and 260 µl of 52% perchloric acid were added to the residual precipitate of sugar solution. The mixture was shaken for 15 min, and then 400 µl of distilled water was added to the mixture and centrifuged at 5000 rpm for 10 min. The supernatant was separated, and 100 µl of cold distilled water and 130 µl of 52% hydrochloric acid were added to the remained residue and centrifuged at 5000 rpm for 10

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min. The supernatant was added to the previous mixture. The mixture was placed in an ice bath for 30 min. Then the mixture was diluted with distilled water to 2 ml. The 50 µl of the mixture solution and 400 µl 2% anthrone were added to the microtubule. The samples were placed in a microplate at 65 °C for 20 min and then transferred to an ice bath. The samples were measured at 630 nm by a microplate spectrophotometer (Epoch, Biotech, USA) [21].

2.3.4. Proline Content

Briefly, 0.1 g of leaves from each treatment was put in a test tube with 10 ml of methanol. The test tubes were placed in a water bath (completely dark condition at 40 °C) for 3 h. The extract passed from Whatman filter paper. Ninhydrin reagent (first, 2.25 g of Ninhydrin was dissolved in 54 ml of glacial acetic acid and 36 ml of 6 M phosphoric acid) and 450 µl of distilled water was added. The 50 µl extract and 250 µl of (Ninehydride reagent, water) were transferred into the microtube and were incubated at 65 °C for 45 min. After removing the samples from the incubator, the samples were immediately cooled. After incubation, 250 µl of the solution was transferred into the microplate. The samples were measured at 515 nm by a microplate spectrophotometer (Epoch, Biotech, USA). Methanol and the reaction mixture were used as blank. The proline content was calculated using the standard curve with L-proline and reported µmol g^{-1} fresh weight [22].

2.3.5. Total Protein Content

Total protein content was determined according to Bradford method. Bradford reagent was prepared by adding 10 ml of 96% ethanol to 0.02 g of Kumasi Brilliant Blue and mixed in the dark condition. Then, 160 ml distilled water added. The 20 ml of 85% phosphoric acid was added to it, and finally, its volume was reached to 200 ml with distilled water. Then, 30 µl of enzyme extract was added to 1.5 ml of Bradford reagent and vortexed. Absorbance measured at 595 nm by spectrophotometer (Epoch, Biotech, USA) [23].

2.3.6. Malondialdehyde (MDA) Content

For MDA content, 0.1 g of leaf tissue was ground in liquid nitrogen with mortar and pestle and removed in microtube and 2 ml of 0.1% trichloroacetic acid (TCA) solution (w/v) was added. Samples were centrifuged at 10,000 rpm for 10 min. The 20 µl supernatant was transferred to a new microtube, and then, 500 µl of 20% TCA and 500 µl of 0.5% thiobarbituric acid (TBA) were added. The samples were placed in a water bath at 95 °C for 30 min and then rapidly transferred to an ice bath. Absorbance was measured at 532 and 600 nm by a spectrophotometer (Epoch, Biotech, USA). The MDA content was expressed as nmol g^{-1} fresh weight [24].

2.3.7. Hydrogen Peroxide (H_2O_2) Content

Measurement of H_2O_2 was performed using potassium iodide (KI). Briefly, 0.1 g of leaf tissue was grounded and powdered with liquid nitrogen into the pestle and mortar. Then 2 ml of 1% TCA was added to the microtube sample and centrifuged at 4 °C at 10,000 rpm for 10 min. The 250 µl of supernatant, 500 µl of 1 M KI, and 250 µl of 10 mM potassium phosphate buffer were added to a microtube and vortexed. The sample absorbance was read at 390 nm. A standard curve by H_2O_2 in 0.1% TCA was used. The H_2O_2 content was reported as µM g^{-1} fresh weight [25].

2.4. Secondary Metabolites

2.4.1. Extraction for Total Phenols, Flavonoid and Total Antioxidant Capacity

To prepare the extraction, 0.1 g of fresh leaf tissue was placed in a test tube containing 10 ml of methanol. The test tubes were placed in hot water at 40 °C for 3 h (Dark conditions). Then, the samples were filtered by the Whatman filter paper. Finally, samples were stored at 4 °C to determine total phenols, flavonoids, and antioxidant capacity.

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2.4.2. Total Phenols

Total phenols content was measured by Folin-Ciocalteau method. Briefly, 200 µl of extract were mixed with 100 µl of 1N Folin. After 5 min, 1000 µl of 2% sodium carbonate was added to the mixture. Samples were stored at room temperature for 30 min in the dark condition. The absorbance was measured at 765 nm by a spectrophotometer (Epoch, Biotech, USA). A standard curve was prepared by gallic acid at different concentrations to calculate the total phenol content. Total phenol was reported as mg gallic acid equivalents (GAE) L^{-1} [26].

2.4.3. Flavonoids

Fifty µl of each sample extract was mixed with 10 µl of 1M sodium acetate and 10 µl of 10% aluminium chloride. Then, 150 µl of methanol was added to the mixture. Samples were kept in the dark place for 40 min. The absorbance was measured at 415 nm by a spectrophotometer (Epoch, Biotech, USA). A standard curve using different quercetin concentrations was used to calculate the flavonoid. Flavonoids were expressed as µg of quercetin per 100 g of fresh weight [27].

2.4.4. Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. 0.1 g of fresh leaf grounded and powdered in liquid nitrogen. Then, 10 ml of methanol was added and homogenized. The mixture was centrifuged at 4 °C for 15 min at 12,000 rpm. The supernatant was collected. The 80 µl of the supernatant was added to 160 µl of 0.004 mg/ml DPPH and kept at room temperature for 30 min. The absorbance was measured at 517 nm by spectrophotometer (Epoch, Biotech, USA), and reported as % of inhibition [28].

2.5. Antioxidant Enzymes Activities

2.5.1. Enzyme Extraction

Antioxidant enzyme extraction was performed according to the method used by Sun et al. [29] with slight modification as follows. The 0.25 g of fresh leaf sample was ground and powdered using mortar and pestle in liquid nitrogen. Then 1 ml of 50 mM potassium phosphate buffer (pH=7) containing 1% polyvinylpyrrolidone (PVP) 1 mM of ethylene diamine tetraacetic acid (EDTA) was added to the samples and centrifuged at 4 °C for 15 min at 12,000 rpm. The supernatant of enzyme extraction was stored at -80 ºC for the measurement of SOD, CAT, and APX enzyme activities.

2.5.2. Superoxide dismutase (SOD) Activity

To measure SOD enzyme activity, 50 µl of enzyme extract was added to 2 ml of the reaction mixture containing 50 mM potassium phosphate buffer (pH=7.8), 13 mM of L-methionine, 75 µM of nitroblue tetrazolium (NBT), 1 mM of EDTA, 2 µM riboflavin. The reaction mixtures were placed under fluorescent lamps for 15 min. Two same mixtures without enzyme extract were kept in the dark and light and used as blank and control. The reaction was stopped by turning off the light and placing the samples in the dark. The absorbance was read at 560 nm, and one-unit SOD activity was determined as the amount of enzyme that caused 50% reduction of NBT. The specific SOD activity was expressed as U mg^{-1} protein [30].

2.5.3. Catalase (CAT) Activity

The CAT enzyme activity was measured by measuring the decomposition of H_2O_2 during one minute at 240 nm with a spectrophotometer (Dynamica, UK). The 3 mL reaction mixture was 50 µl of enzymatic extract, 2900 µl of 50 mM potassium phosphate buffer (pH=7), 50 µl of 0.1 mM H_2O_2. One unit was defined as decomposition of H_2O_2 changes per min. The specific CAT activity was expressed as U mg^{-1} protein [29].

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2.5.4. Ascorbate Peroxidase (APX) Activity

The APX enzyme activity was measured using a spectrophotometer (Dynamica, UK) at 290 nm. The reaction mixture was 2900 µl of 50 mM potassium phosphate buffer (pH=7), 50 µl of 0.1 mM H_2O_2., and 50 µl of the enzyme extract. The enzyme activity was expressed as one milligram of ascorbic acid oxidation per minute to one milligram of protein. The extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to evaluate enzyme activity [29].

2.6. Statistical Analysis

A factorial experiment was set up in a complete randomized design (CRD) with 16 treatments (4 salinity levels and 4 bacterial levels). Statistical analysis of the data was performed using R software, and the comparison of the means was performed using the LSD test at 5% level of probability.

3. Results

3.1. Salinity Effects on The Morphophysiological Traits of Purple Basil

3.1.1. Plant Growth Parameters

Treating purple basil with different NaCl concentrations (control (0 mM), S1 (50 mM), S2(100 mM), and S3 (150 mM)), showed the significant effects on fresh weight (FW), dry weight (DW), and leaf area index (LAI) (Table 1). Fifty mM NaCl had a positive impact on plant growth parameters, however 100 and 150 mM NaCl represented the negative effect on indicated factor. DW and FW were significantly higher at 50mM NaCl but no significant changes were observed on LA compare to control. FW, DW, and LA significantly decreased at 100mM and 150 mM NaCl concentrations compared to the control group. Therefore, the salinity toxic effects were observed on plant growth parameters at moderate (S2) and high (S3) NaCl concentrations (

Table **1**).

Table 1. Difference in dry weight (DW), fresh weight (FW) and leaf area index (LAI) after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a, b and c indicate the Post hoc LSD Test)

	DW		FW		LAI	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.13±0.02	<0.001 [a]	2.25±0.43	<0.001 [b]	12.97± 1.38	<0.001 [c]
S1(50 mM)	0.20±0.02		3.00±0.37		13.49± 0.39	
S2(100 mM)	0.09±0.01		1.19±0.13		8.89± 0.79	
S3(150 mM)	0.08±0.01		0.79±0.20		7.43± 0.90	

a: P_1=0.001, P_2=0.005, P_3=0.002, P_4<0.001, P_5<0.001, P_6=0.636

b: P_1=0.018, P_2=0.003, P_3<0.001, P_4<0.001, P_5<0.001, P_6=0.154

c: P_1=0.515, P_2=0.001, P_3<0.001, P_4<0.001, P_5<0.001, P_6=0.092

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3 vs. S2

3.1.2. Biochemical Changes

3.1.2.1. Chlorophylls (a, b, Total) and Carotenoids Content

There was no significant difference in chlorophyll *a* level at 100 and 150 mM NaCl in comparison with the control group. However, at the 50 mM salinity level, chlorophyll *a* (17.28%) decreased compared to the control group (Table 2). The results showed that increasing salinity levels caused no significant difference on chlorophyll *b* in purple basil. The same result was observed in total chlorophyll levels. However, salinity significantly affected carotenoid content. These effects were highest at 150 mM NaCl concentration (

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Table 2).

Table 2. Difference in Chlorophyll a (Chl.a), Chlorophyll b (Chl.b), Total chlorophyll (Chl. Total), and Carotenoid after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	Chl.a		Chl.b		Chl.T		Carotenoid	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.82±0.05	0.016[a]	0.18±0.01	0.157	0.99±0.06	0.421	0.27±0.02	0.024[b]
S1(50 mM)	0.67±0.07		0.16±0.02		0.95±0.14		0.26±0.01	
S2(100 mM)	0.81±0.03		0.17±0.01		0.89±0.09		0.26±0.02	
S3(150 mM)	0.81±0.05		0.19±0.01		1.01±0.05		0.31±0.02	

a: P_1=0.006, P_2=0.877, P_3=0.857, P_4=0.008, P_5=0.006, P_6=0.988

b: P_1=0.283, P_2=0.311, P_3=0.033, P_4=0.950, P_5=0.008, P_6=0.008

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3 vs. S2

3.1.2.2. Soluble Carbohydrates, Starch, Proline and Total Protein Contents

The aerial parts' soluble carbohydrates significantly decreased in different salinity levels compared to the control group. Starch and Proline levels also follow the same pattern. However, a dose response relationship could not be observed between different salinity levels. Total protein content showed no definite pattern in different groups (

Table 3).

Table 3. Difference in SC (Soluble Carbohydrates), Starch, Proline, and Protein content after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a, b, c indicate the Post hoc LSD Test)

	SC		Starch		Proline		Protein	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	88.88± 1.87	0.011[a]	89.96± 1.49	0.18	1073.79± 58.72	0.004[b]	0.57±0.05	0.003[c]
S1(50 mM)	82.73± 3.10		84.04± 4.92		1567.79± 104.70		0.52±0.06	
S2(100 mM)	79.80± 1.35		87.46± 2.47		1575.03± 163.34		0.72±0.01	
S3(150 mM)	83.27± 2.90		88.71± 2.20		1820.22± 275.87		0.62±0.05	

a: P_1=0.014, P_2=0.002, P_3=0.022, P_4=0.175, P_5=0.794, P_6=0.117

b: P_1=0.010, P_2=0.009, P_3=0.001, P_4=0.963, P_5=0.109, P_6=0.118

c: P_1=0.256, P_2=0.003, P_3=0.154, P_4=0.001, P_5=0.019, P_6=0.022

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3 vs. S2

3.1.2.3. Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2) Content

MDA and H2O2 content were affected by salinity stress. But these changes did not follow a dose response relationship. These results are shown in

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Table 4.

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Table 4. Difference in MDA (Malondialdehyde) and H_2O_2 (Hydrogen Proxide) after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	MDA		H2O2	
	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	2.37±0.12	0.003^{a}	15.21± 0.12	0.026^{b}
S1	2.37±0.05		15.78± 0.23	
S2	2.20±0.61		15.53± 0.08	
S3	3.63±0.24		15.70± 0.26	

a: P_1=0.998, P_2=553, P_3=0.002, P_4=0.554, P_5=0.002, P_6=0.001

b: P_1=0.007, P_2=0.083, P_3=0.011, P_4=0.154, P_5=0.605, P_6=0.288

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3

3.1.3. Secondary Metabolites

By increasing salinity levels, the total phenols content had no significant difference with the control group. The results showed that by increasing salinity levels, flavonoids content significantly increased compared to the control. Total antioxidant capacity (TAC) was affected by salinity stress as well. For flavonoid and TAC, a partial dose response relationship could be observed (

Table 5).

Table 5. Difference in Phenol, Felavenoid and Total Antioxidant Capacity (TAC) after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	Phenol		Flavonoid		TAC	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.02±0.00	0.208	734.09± 67.17	$<0.001^{a}$	31.27± 0.70	$<0.001^{b}$
S1	0.03±0.00		1256.34± 154.20		49.85± 3.01	
S2	0.02±0.00		1219.41± 135.48		47.74± 4.99	
S3	0.03±0.00		1536.17± 87.66		53.00± 0.60	

a: P_1<0.001, P_2<0.001, P_3<0.001, P_4=0.696, P_5=0.014, P_6=0.007

b: P_1<0.001, P_2<0.001, P_3<0.001, P_4=0.408, P_5=0.227, P_6=0.061

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3 vs. S2

3.1.4. Antioxidant Enzymes Activity

The SOD activity had not changed by the salinity stress. On the other hand, CAT activity significantly increased compared to the control group by a partial dose response relationship. APX activity significantly decreased with a partial dose response relationship (

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Table 6).

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Table 6. Difference in SOD (superoxide dismutase), CAT (Catalase) and Ascorbate peroxidase (APX) after the treatment with three salinity levels (S1-3) in purple basil. (Analysis of variance (AOV) test. a indicates the Post hoc LSD Test)

	SOD		CAT		APX	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	121.30± 7.42	0.241	0.56±0.08	<0.001[a]	32.75± 5.25	<0.001[b]
S1	116.77± 8.44		1.40±0.17		17.83± 2.03	
S2	125.47± 14.69		1.36±0.09		26.42± 2.00	
S3	132.25± 6.50		1.20±0.26		43.50± 2.59	

a: $P_1<0.001$, $P_2<0.001$, $P_3<0.001$, $P_4=0.761$, $P_5=0.138$, $P_6=0.252$

b: $P_1=0.001$, $P_2=0.044$, $P_3=0.004$, $P_4=0.012$, $P_5<0.001$, $P_6<0.001$

P_1: S1 vs. Control, P_2: S2 vs. Control. P_3: S3 vs. Control, P_4: S2 vs. S1, P_5: S3 vs. S1, P_6: S3 vs. S2

Altogether, results of the first section showed that 50mM NaCl has a positive impact on plant growth parameters and therefore it is not stressful for the purple basil. On the other hand, purple basil faced to the moderate and severe stressful conditions at 100mM and 150mM NaCl respectively. Among the measured factors, the negative spearman correlation was observed between plant growth parameters (FW, DW and LA) and H2O2, proline content, MDA, CAT, TAC, Flavonoid, APX and Carotenoid under 150mM saline condition (Figure 1).

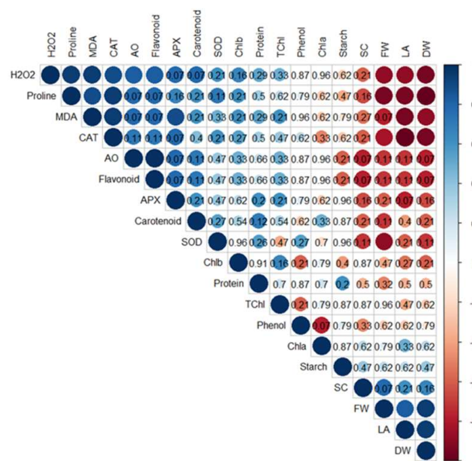

Figure 1- Spearman correlation plot with P-value > 0.05 are considered as insignificant. Colored circle without number represented a significant correlation.

3.2. Effects of Azotobacter Sp. and Halomonas Sp. on the Morphophysiological Traits of Purple Basil

3.2.1. Plant Growth Parameters

Treating purple basil with bacteria including *Azotobacter* sp. (A) and *Halomonas* sp. (H), did not show any significant effects on fresh weight (FW), dry weight (DW), and leaf area index (LAI). However, the mixture of *Azotobacter* sp. and *Halomonas* sp. (A+H) had a negative effect on FW (

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Table 7).

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Table 7. Difference in dry weight (DW), (fresh weight (FW) and leaf area index (LAI) after the treatment with Azotobacter sp. (A), Halomonas sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a indicates the Post hoc LSD Test)

	DW		FW		LAI	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.13±0.02	0.913	2.25±0.43	0.048[a]	12.97±1.38	0.309
A	0.13±0.01		2.13±0.18		11.13±0.78	
H	0.14±0.02		2.47±0.20		12.16±0.69	
A+H	0.13±0.03		1.65±0.29		12.07±1.35	

a: P_1=0.630, P_2=0.380, P_3=0.038, P_4=0.191, P_5=0.084, P_6=0.009

P_1: Control vs. A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H, P_6: H vs. A+H

3.2.2. Biochemical Changes

3.2.2.1. Chlorophylls (a, b, total) and Carotenoids Content

The treatment of purple basil separately with *Azotobacter* sp or *Halomonas* sp did not have convincing results on all measured chlorophylls. Interestingly *Azotobacter*+*Halomonas* had significant results on these measures compared to the control group. Post hoc tests also confirmed that the mixture of *Azotobacter* sp and *Halomonas* sp significantly increases the measured chlorophylls. Result also showed that there were no changes on carotenoid with different treatments (

Table 8).

Table 8. Difference in Chlorophyll a (Chl.a), Chlorophyll b (Chl.b), Total Chlorophyll (Chl. Total) and Carotenoid after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus Halomonas sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a, b and c indicate the Post hoc LSD Test)

	Chl.a		Chl.b		Chl.T		Carotenoid	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.82±0.05	<0.001[a]	0.18±0.01	<0.001[b]	0.99±0.06	<0.001[c]	0.27±0.02	0.419
A	0.63±0.08		0.18±0.01		0.84±0.06		0.25±0.02	
H	0.69±0.04		0.19±0.01		0.94±0.08		0.26±0.01	
A+H	1.04±0.08		0.25±0.01		1.29±0.09		0.27±0.03	

a: P_1=0.010, P_2=0.048, P_3=0.004, P_4=0.328, P_5<0.001, P_6<0.001

b: P_1=0.850, P_2=0.242, P_3<0.001, P_4=0.317, P_5<0.001, P_6<0.001

c: P_1=0.032, P_2=0.417, P_3=0.001, P_4=0.121, P_5<0.001, P_6<0.001

P_1: Control vs A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H, P_6: H vs. A+H

3.2.2.2. Soluble carbohydrates, Starch, Proline and total protein contents

Priming the purple basils with bacteria represented in a significant increase in protein content in all treatments compare with control. *Halomonas* sp shows more promising increase than *Azotobacter* sp regarding Proline levels. On the other hand, *Azotobacter*, *Halomonas* or *Azotobacter*+*Halomonas* had negatively affected the soluble carbohydrate (SC). Among the treatments, only *Azotobacter* sp. decreased the starch amount in the purple basil (

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Table 9).

Table 9. Difference in SC (Soluble Carbohydrates), Starch, Proline, and Protein content after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a, b and c indicate the Post hoc LSD Test)

	SC		Starch		Proline		Protein	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	88.18±1.87	<0.001[a]	89.96±1.49	<0.015[b]	1073.79±58.72	<0.008[c]	0.57±0.05	0.004[d]
A	82.58±1.38		81.43±4.36		1516.22±313.34		0.69±0.01	
H	86.22±1.45		86.58±0.95		1542.46±41.11		0.66±0.04	
A+H	77.47±1.08		91.08±3.47		1812.08±218.68		0.70±0.01	

a: P_1=0.001, P_2=0.043, P_3<0.001, P_4<0.010, P_5<0.002, P_6<0.001

b: P_1=0.007, P_2=0.194, P_3=0.650, P_4=0.063, P_5=0.004, P_6=0.096

c: P_1=0.235, P_2=0.014, P_3=0.091, P_4=0.100, P_5=0.543, P_6=0.256

d: P_1=0.002, P_2=0.008, P_3=0.001, P_4=0.364, P_5=0.626, P_6=0.180

P_1: Control vs A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H, P_6: H vs. A+H

3.2.2.3. Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2) Content

MDA levels were significantly decreased by Azotobacter sp. or Halomonas sp. separately but not together. Only Halomonas sp (and not Azotobacter sp or Azotobacter sp+Halomonas sp) decreased H2O2 content in purple basil (

Table 10).

Table 10. Difference in MDA (Malondialdehyde) and H_2O_2 (Hydrogen Proxide) after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	MDA		H2O2	
	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	2.37±0.12	<0.001[a]	15.21±0.12	<0.002[b]
A	1.61±0.07		15.48±0.48	
H	1.83±0.13		14.01±0.37	
A+H	2.31±0.12		14.78±0.13	

a: P_1<0.001, P_2<0.001, P_3=0.526, P_4=0.040, P_5<0.001, P_6<0.001

b: P_1=0.339, P_2=0.002, P_3=0.130, P_4<0.001, P_5=0.027, P_6=0.018

P_1: Control vs A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H

3.2.3. Secondary Metabolites

It seems to be able to decrease the total phenols contents and increase the Flavonoid content in purple basil. None of the treatments significantly affected the total antioxidant capacity (

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Table 11).

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Table 11. Difference in Phenol, Flavonoid and Total Antioxidant Capacity (TAC) after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	Phenol		Flavonoid		TAC	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.02±0.0013	<0.001[a]	734.09±67.17	<0.001[b]	31.27±0.70	0.073
A	0.02±0.0017		1257.77±73.43		28.84±1.34	
H	0.03±0.0007		812.22±167.66		25.31±3.64	
A+H	0.02±0.0007		1079.73±70.66		27.63±2.46	

a: P_1=0.003, P_2=0.111, P_3=0.001, P_4<0.001, P_5=0.571, P_6<0.001

b: P_1<0.001, P_2=0.334, P_3=0.001, P_4<0.001, P_5=0.058, P_6=0.010

P_1: Control vs A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H, P_6: H vs. A+H

3.2.4. Antioxidant Enzymes Activity

It seems to decrease SOD activity and increase the APX activity. All of the treatments increased the CAT activity in purple basil (

Table **12**).

Table 12. Difference in SOD (superoxide dismutase), CAT (Catalase) and Ascorbate peroxidase (APX) after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) in purple basil. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	SOD		CAT		APX	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	121.30±7.42	<0.001[a]	0.56±0.08	<0.001[b]	32.75±5.25	<0.04[c]
A	72.81±4.87		1.39±0.03		39.47±4.97	
H	120.00±9.54		1.11±0.10		32.31±2.39	
A+H	72.34±3.14		0.97±0.18		29.24±0.80	

a: P_1<0.001, P_2=0.818, P_3<0.001, P_4<0.001, P_5=0.934, P_6=0.818

b: P_1<0.001, P_2<0.001, P_3=0.001, P_4=0.012, P_5=0.001, P_6=0.149

c: P_1=0.054, P_2=0.896, P_3=0.307, P_4=0.043, P_5=0.008, P_6=0.368

P_1: Control vs A, P_2: Control vs. H. P_3: Control vs. A+H, P_4: A vs. H, P_5: A vs. A+H

Section 2 shows that *Azotobacter* sp and *Halomonas* sp treatment did not have any significant changes on purple basil growth parameters. In both salinity and treatment with the selected bacteria significant increase in Proline and Catalase were observed. However, the response of purple basil was opposite in the treatment with the selected bacteria and salinity stress concerning the MDA content. To sum up, *Azotobacter* sp and *Halomonas* sp potentially can increase the tolerance of purple basil under salinity stress. As the most effects were seen at the highest tested salinity concentration (150 mM NaCl) and *Azotobacter* sp., the next section summarizes the related results to find out if *Azotobacter* sp. could increase the tolerance of purple basil at 150 mM NaCl.

3.3. The Effects of Azotobacter sp. on the Salinity Stress in Purple Basil

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Plant growth parameters (dry weight (DW), fresh weight (FW) and leaf area index (LAI)) were measured after the treatment with *Azotobacter* sp, *Halomonas* sp or *Azotobacter* sp +*Halomonas* sp on purple basil. 150 mM NaCl salinity stress was imposed to all groups. Result showed that in *Azotobacte*r sp, *Halomonas* sp and *Azotobacter* sp +*Halomonas* sp groups, all of the measured growth parameters were significantly lower than control. And compared to *Halomonas* sp, *Azotobacter* sp was more effective to ameliorate the salt stress (

Table **13**).

Table 13. Difference in dry weight (DW), fresh weight (FW) and leaf area index (LAI) after the treatment with *Azotobacter* sp. (A), *Halomonas* sp. (H) or *Azotobacter* sp. plus *Halomonas* sp. (A+H) on purple basil. 150 mM NaCl salinity stress was imposed to all groups. (Analysis of variance (AOV) test. a and b indicate the Post hoc LSD Test)

	DW		FW		LAI	
	Mean±Sd	P-Value	Mean±Sd	P-Value	Mean±Sd	P-Value
Control	0.1339±0.0211	<0.001[a]	2.25±0.4321	<0.001[b]	12.9734±1.3813	<0.001[c]
S3	0.0825±0.0075		0.7942±0.1956		7.4329±0.8982	
S3+A	0.1642±0.0194		1.7975±0.0855		9.3376±0.5103	
S3+A+H	0.0883±0.0104		1.1883±0.1173		9.3224±1.1263	
S3+H	0.0672±0.0118		0.7678±0.1078		7.7166±0.8222	

a: P_1=0.002, P_2=0.033, P_3=0.004, P_4<0.001, P_5<0.001
P_6=0.644, P_7=0.241, P_8<0.001, P_9<0.001, P_{10}=0.116

b: P_1<0.001, P_2=0.035, P_3<0.001, P_4<0.001, P_5<0.001
P_6=0.059, P_7=0.890, P_8=0.008, P_9<0.001, P_{10}=0.047

c: P_1<0.001, P_2=0.001, P_3=0.001, P_4<0.001, P_5=0.041
P_6=0.042, P_7=0.733, P_8=0.985, P_9=0.073, P_{10}=0.076

P_1: S3 vs. Control, P_2: S3+A vs. Control. P_3: S3+A+H vs. Control, P_4: S3+H vs. Control, P_5: S3+A vs. S3
P_6: S3+A+H vs. S3, P_7=S3+H vs S3, P_8=S3+A+H vsS3+A, P_9=S3+H vs S3+A, P_{10}=S3+H vs S3+A+H

S3+*Azotobacter* had shown to increase SC, protein and proline content compared to the control group. However, the amount of starch did not change significantly among the treatment groups. Interestingly SC and protein content were higher in the S3+*Azotobacter* group compared to the S3 group (Figure 2). Moreover, MDA and H2O2 represented a lower value in the S3+*Azotobacter* group compared to the S3 group. Flavonoid content was significantly higher in both S3 and S3+*Azotobacter* groups compared to the control group. On the other hand, the total antioxidant capacity (TAC) in the S3+*Azotobacter* sp group was the same as the control group and was lower than the S3 group (Figure 3). Regarding the antioxidant enzymes, the S3+*Azotobacter* treatment group got the highest value in catalase and the lowest value in SOD measurements. Result showed that S3 and S3+*Azotobacter* groups increased the level of APX compared to the control group (Figure 4).

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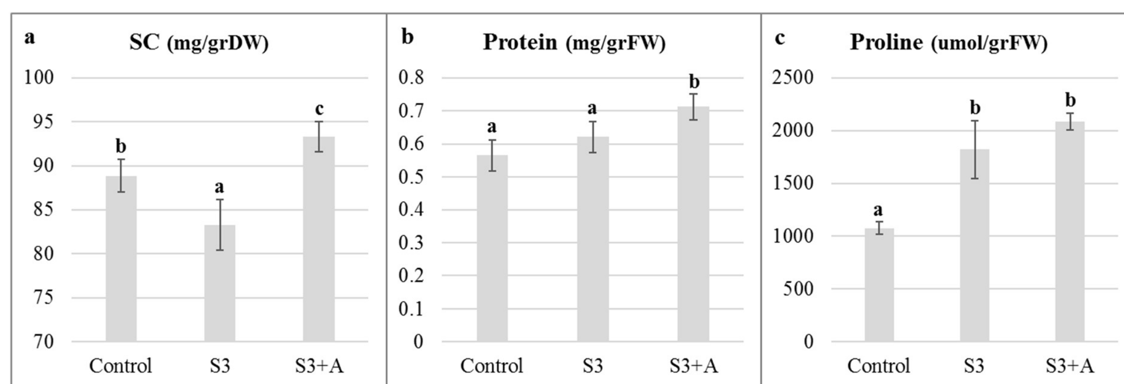

Figure 2. The effects of 150 mM NaCl salinity stress (S3) and *Azotobacter* sp. (A) on soluble carbohydrate (SC) (a), protein (b) and proline (c) of purple basil. Error bars indicate standard deviation (n = 3). Different lowercase letters denote statistical differences between treatment groups at the 5% level according to LSD's test.

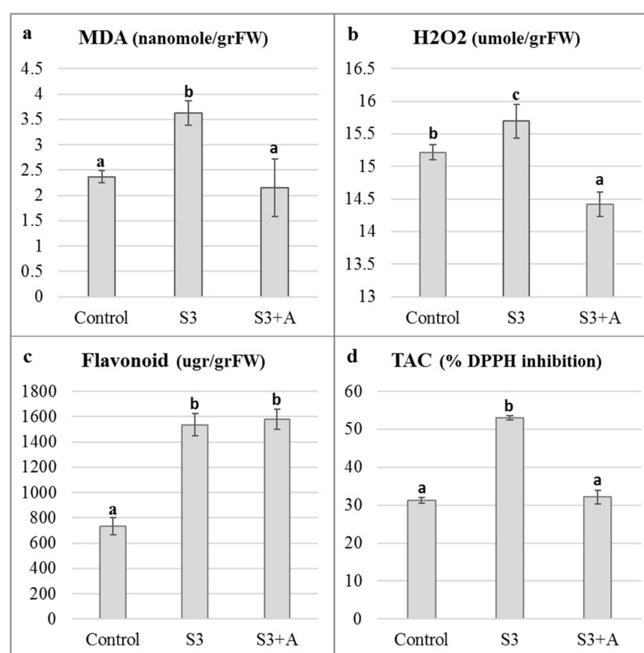

Figure 3. The effects of 150 mM NaCl salinity stress (S3) and *Azotobacter* sp. (A) on Malondialdehyde (MDA) (a), H2O2 (b), Flavonoid (c) and Total antioxidant capacity (TAC) (d) of purple basil. Error bars indicate standard deviation (n = 3). Different lowercase letters denote statistical differences between treatment groups at the 5% level according to LSD's test.

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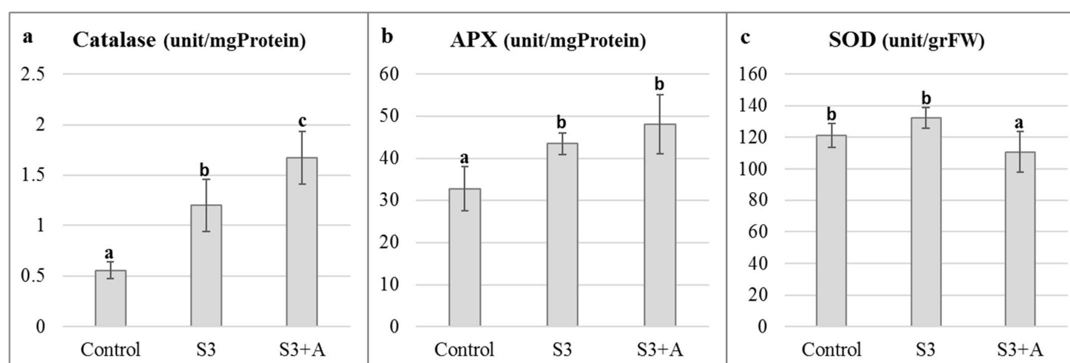

Figure 4. The effects of 150 mM NaCl salinity stress (S3) and *Azotobacter* sp. (A) on Catalase (a), Ascorbate peroxidase (APX) (b), and Superoxide dismutases (SOD) (c) of purple basil. Error bars indicate standard deviation (n = 3). Different lowercase letters denote statistical differences between treatment groups at the 5% level according to LSD's test.

4. Discussion

The results showed that purple basil showed different responses to bacteria inoculation under salinity stress. In general, growth parameters significantly decreased by increasing salinity levels from 50 to 100 and 150 mM NaCl. It has been reported that basil plants can manage growth under saline conditions [31]. Also the reports show that the tolerance of two basil cultivars to salt stress depends on the cultivar and salinity levels [32]. These results agree with our findings, showing that adverse effect of salinity on growth parameters of purple basil can be observed at 100 and 150 mM NaCl. Growth changes in basil plants are associated with physiological dehydration due to the increased osmotic pressure under saline conditions, which can reduce the plant's ability for water uptake. Also, under salinity stress, high sodium chloride concentration can enter the leaf cells by transpiration and reduce the photosynthesis rate and plant growth. In addition, it has been shown that photosynthesis level decreases under salinity stress due to reduce stomatal conductance and carbon dioxide (CO_2) diffusion across the mesophyll. Besides, growth reduction under salinity conditions may decrease cytoplasmic volume of the plant and cause the loss of cellular turgor [33].

Our results showed that *Halomonas* sp had no significant effect on purple basil growth at the 150 mM NaCl. Nevertheless, *Azotobacter* sp and a combination of *Halomonas* sp and *Azotobacter* sp (A+H) improved the growth parameters at 150 mM NaCl. It can be because of the different modes of action of bacteria, the different interactions of bacteria with plants, and the tested salinity levels. In general, PGPR's use had shown to improve the growth parameters of plants compared to a non-inoculated plant under the same stress conditions. Growth improvement under stress conditions has been reported to be due to the amelioration of the adverse effects of salt stress [34]. Therefore, salt-tolerant bacteria can have better water and mineral uptake by producing microbial and hormonal metabolites such as Auxin and Cytokinin, which subsequently can increase the plant tolerance against salinity stress [35]. *Azotobacter* sp. involves in producing Auxin, promoting cell division and elongation [36]. *Azotobacter* sp. Had shown to fix nitrogen, dissolve phosphorus and potassium and increase nutrient availability in tomato [37].

Increasing potassium transport through xylem and changing the Na^+ movement from leaf to roots is an excellent solution for improving a plant's tolerance to stress. PGPRs can promote 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, indole acetic acid (IAA), siderophore, and phosphate solubility that increase growth, yield, and plant tolerance to salt stress. ACC deaminase enzyme is involved in increasing available phosphorus, potassium uptake, and plant hormone synthesis, which is useful for enhancing plant growth under saline conditions [38]. In addition, PGPRs play a vital role in increasing aerial parts' fresh and dry weight in the inoculated plant under stress conditions, which are in accordance with our results.

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Under salt stress conditions, chlorophyll *a*, *b*, and total chlorophyll did not change at 100 and 150 mM NaCl levels. However, carotenoids significantly increased at 150 mM NaCl level between the applied treatments. It has been reported that total chlorophyll can be increased at 25 mM NaCl levels, while total chlorophyll were increased at 50 mM NaCl levels [39].

Salt-resistant plants have higher chlorophyll contents compared to the sensitive plants. Therefore, changes in chlorophyll contents can use as a biochemical indicator for salinity tolerance in plants. However, it has also been reported that increasing chlorophyll content from 0 to 130 mM NaCl has inhibition effects on morphological parameters. It might indicate that changes in chlorophyll are not a good indicator for salinity resistance in basil plant [40]. In our study, salt stress had no significant effect on photosynthetic pigments, which shows the ability of the plant for adaption under salinity stress due to the gradual increase of NaCl concentrations. Other research on purple basil also showed that salinity stress did not affect chlorophyll content [41].

Carotenoids play essential roles in plants. They can act as antioxidants, photosynthetic pigments, pollinators, and seed dispersers. Production of ROS, especially singlet oxygen, in chloroplasts under stress conditions can oxidize carotenoids and convert them to some sub-products, like aldehydes, ketones, endoperoxides, and lactones [42]. A study showed that abiotic stress can lead to the accumulation of carotenoids as occurred in potato mini-tubers under drought stress by polyethylene glycol 4000 [43]. On the contrary, in our study, the bacterial inoculation had no significant effect on carotenoids content.

To prevent cellular damage under oxidative stress, plants accumulate soluble substances named osmolytes to protect their cells. The most common osmolytes in regulation osmosis are proline, glycine betaine, polyamines, proteins, and sugars. These compounds stabilize the osmotic pressure between the cell and the cytosol [44]. Under stress conditions, plants can balance the osmotic pressure by the accumulation of soluble sugars in the cells [45]. In our study, soluble sugars significantly decreased under salinity stress, which was in agreement with previous studies on basil cultivars [46]. Accumulation and production of osmolytes by PGPR are being conducted by mechanisms that protect the plant against salinity stress [47]. In our study, *Azotobacter* sp treatment had the highest soluble sugars content at 150 mM NaCl.

In our study, total protein content had no significant difference between treatment and control groups under salinity stress conditions. It has been reported that the effect of salinity stress on protein content depends on NaCl concentrations. On the other hand, protein content can be increased at a lower level of NaCl, but at a higher level of NaCl, the protein content in the stems and leaves could be reduced. In a study it is shown that the total protein content can be decreased in sweet basil leaves under 300 mM NaCl salinity stress [48]. However, another study reported that the total protein content can be even increased in basil at 80 mM NaCl [49]. Based on obtained results, it can be suggested that the response of basil to salinity stress depends on the cultivar and NaCl concentrations. In a study, *Azotobacter* sp. treatment caused the accumulation of protein in the tomato [37], which increased plant resistance to salinity stress and increased plant osmotic regulation[50], and maintained plant water balance [47]. These results agree with our findings and indicated that *Azotobacter* sp. treatment had caused protein accumulation at 150 mM NaCl.

Proline content can be accelerated by increasing the salinity level to 150 mM NaCl. *Azotobacter* sp, *Halomonas* sp, and *Azotobacter+Halomonas* treatments showed a significant enhancement on proline content compared to the control group. Increasing proline content has been associated with cell osmosis regulation, protecting the photosynthetic systems, increasing nitrogen fixation, and reducing ROS production, which can reduce the adverse effect of salinity stress due to oxidative damage [51]. Halophilic bacteria increased the of proline expressing genes such as *pro J*, *pro H*, and *pro A*, resulting in the accumulation of proline in plants under salinity stress [52]. Moreover, it has been reported that

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inoculation of basil with *Azotobacter* increased proline content, improved photosynthesis, water uptake, and water use efficiency [53].

Under oxidative stress, some biomarkers such as MDA and H_2O_2 are associated with ion leakage [54]. MDA had a significant increase at 150 mM NaCl, but *Azotobacter* sp. treatments had no significant effects on MDA content compared to the control group at different salinity levels. Plant inoculated with microorganisms can reduce ROS accumulation (H_2O_2, single oxygen, hydroxyl radical, and superoxide) and protect cell membranes from oxidative damage [55]. Other studies showed that MDA content can increase by 30.18% in the tomato compared to the control group under salinity stress. However in that study, tomato inoculated with *Azotobacter* sp. reduced MDA content under salt stress conditions [37].

H_2O_2 is one of the most stable types of ROS produced in response to many physiological stimuli in plant cells and causes oxidative stress and subsequently protein inactivation [56]. In general, H_2O_2 content in purple basil had no significant difference at different salinity levels. However, bacterial treatments reduced H_2O_2 content, but this reduction is dependent of NaCl concentrations. For example, *Azotobacter* sp. treatment at 150 mM NaCl showed a significant reduction in H_2O_2 content compared to the control group. Based on the obtained results, it can be suggested that *Azotobacter* sp. can reduce the production of H_2O_2 and thus protect the lipid membrane against peroxidation. This result agrees with recent reports on reducing H_2O_2 content in inoculated plants with root fungi [57] or other endophytic bacteria under salinity stress conditions [36].

Antioxidant activity is mainly related to phenolic compounds such as (flavonoids, phenolic acid, tannins, and phenolic diphtheria) in plants. These phenolic compounds play an essential role in the neutralization of free radicals, single oxygen, and decomposition of peroxides [58,59]. According to our results, by increasing salinity levels, flavonoids and antioxidant capacity were increased, while the total phenols had no significant difference with the control group. Another study reported that by increasing salinity stress, the total phenol increased in the root, but no changes were observed in the young leaves of the corn [60].

According to our results, *Azotobacter* sp treatment significantly had a higher flavonoid content compared to the control plant group at 150 mM NaCl level, while phenol and antioxidant capacity did not change. Another study showed that the higher flavonoids accumulation in the inoculated plant with *Azotobacter* sp plays a vital role in scavenging toxic radicals under salinity stress [55]. Overall, we observed a significant reduction in antioxidant activity in purple basil leaves treated with bacteria at 150 mM NaCl. One of the reasons for the reduction of leaf antioxidant activity in the conditions mentioned above is a decrease in ethylene in plant roots due to the production of ACC deaminase enzyme by these bacteria. Another reason could be the production of these bacteria's antioxidant enzymes and thus no more need in the production of these enzymes in the leaves.

Salinity stress causes ROS generation, severely damaging the cell membrane structure [61]. However, the antioxidant defense system is activated under oxidative stress. This system consists of several enzymes that scavenge the ROS, including CAT, APX, and SOD. These antioxidant enzymes can remove free radicals produced under abiotic stress conditions. The role of antioxidant enzymes as the elements of the tolerance mechanisms involve in response to salt stress. SOD enzymes catalyze the superoxide anion to H_2O_2 [62]. Then, CAT and APX enzymes decompose H_2O_2 and convert it to water molecules. Therefore, under stress conditions, antioxidant enzymes can be increased to scavenge the ROS and reduce the oxidative damage [63].

Our results showed that CAT and APX enzyme activities were induced at a 150 mM salinity level. However, SOD activity had not significantly changed by increasing salinity levels. Among treatments, we observed different activities of SOD, APX, and CAT enzymes. For example, SOD activity had no significant difference at 150 mM NaCl in *Azotobacter* sp. treatment compared the control group. However, APX activity had increased in

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Azotobacter sp. and *Halomonas* sp. treatments, but *Azotobacter+Halomonas* had no significant difference with the control group. In addition, the CAT enzyme showed the highest activity in *Azotobacter* sp treatment. It has been reported that salinity stress increased SOD, CAT and APX activities in the tomato plant, and inoculated plant has the highest antioxidant enzyme activities [64]. Another study showed that the inoculation of *Artemisia annua* with *Azotobacter chroococcum* can increase the antioxidant enzyme activities, including SOD, APX, and CAT under salinity stress. The authors suggested that H_2O_2 can be rapidly removed in the inoculated plant by *Azotobacter* sp. due to an increase in CAT and APX enzyme activities [55]. Our results showed that inoculated plants with *Azotobacter* sp. have shown to reduce H_2O_2 content by increasing CAT and APX enzyme activities. Increasing activities of antioxidant enzymes in inoculated plants with PGPR may be due to the effect on genes expressing antioxidant enzymes under salinity stress [65]. Furthermore, other studies suggest that *Azotobacter* sp. can produce ACC deaminase enzymes [66]. On the other hand, ACC deaminase enzyme can increase APX, CAT, and SOD gene expression under salinity stress. Therefore, inoculated plants with PGPR can produce antioxidant enzymes that enhance plant tolerance to salinity stress [67].

5. Conclusions

The results of this study showed that the levels of some osmolytes and oxidative stress biomarkers, some secondary metabolites, and antioxidant enzymes can be increased under salinity stress. The application of PGPR affects antioxidant enzymes (CAT and APX) and some secondary metabolites such as flavonoids, increasing the osmolytes, and reducing the H_2O_2 in the leaves and subsequently enhanced tolerance the purple basil tolerance to salinity stress. *Azotobacter* sp inoculation at 150 mM NaCl, can reduce the adverse effects of salinity stress by increasing antioxidant enzyme activities and increasing some secondary metabolites, preventing the formation of ROS, and oxidative damage in the purple basil plant. Overall, our findings revealed that *Azotobacter* sp. improved the purple basil tolerance to salinity stress at 150 mM NaCl.

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