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Effect of Exopolysaccharide-Producing Bacteria and Melatonin on Faba Bean Production in Saline and Non-Saline Soil

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Abstract: Soil salinity is a major threat to modern agriculture, as it affects crop growth and development. The present study focuses on the integration of eco-friendly biostimulants in salinity stress as a strategy to achieve the alleviation of abiotic stress. Field experiments were conducted at two locations, consisting of saline and non-saline soil, to investigate the utilization of exopolysaccharide (EPS)-producing bacteria (*Azotobacter chroococcum*) and melatonin at different concentrations (0, 25, 50, and 100 µM) for alleviating the adverse effects of salinity on the growth and production of faba bean plants. Salinity stress caused a reduction in all measured parameters of the faba bean plants grown in the saline soil relative to the plants grown in the non-saline soil. The addition of bacteria and/or melatonin significantly increased the growth parameters and yield components under both soils compared to the respective control plants. Both bacteria inoculation and melatonin application enhanced N, P, and K concentrations; the proline content; RWC%; and the K^+/Na^+ ratio; however, Na^+ and Cl^- concentrations were decreased significantly in salt-stressed faba beans. The combined use of bacteria and melatonin exhibited the highest stimulating effects. The present study recommends the combined use of EPS-producing bacteria and melatonin for the salinity stress management strategy of faba bean.

Keywords: soil salinity; faba bean; biostimulants; EPS-producing bacteria; melatonin

1. Introduction

Salinity stress is one of the most prevalent abiotic stresses and results in significant losses in agricultural crop production, particularly in arid and semi-arid areas [1]. It is estimated that every year, 1.5 million hectares of irrigated land is falling outside of agricultural production and more than 50% of all arable land will suffer from salinization until the year 2050 [2]. Salinity causes osmotic stress and ionic toxicity that lead to oxidative stress in plants [3] and adversely affects the physiology, growth, and yield of crops [4].

Globally, faba bean (*Vicia faba* L.) represents a large part of the human diet [5,6]. It is known that leguminous crops are sensitive to soil salinity all over the world. Salinity decreases the growth and yield of faba bean [7]. Recently, in Egypt, there has been a tendency to extend the cultivated area of several crops, including *Vicia faba*, in newly reclaimed soils, which are often salt-affected soils.

As noted in [8], "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content". Biostimulants enhance the adaptation of plants for maintaining sustainable crop production and improving the soil fertility and structure [9]. Biostimulants are considered a

promising approach for stress management [10]. Melatonin (N-acetyl-5-methoxytryptamine) is an effective biostimulant used to enhance the alleviation of stress; it is a nontoxic biological molecule, found naturally in the animal and plant kingdoms [11]. Many studies have dissected the beneficial roles of melatonin in plant protection [12]. Melatonin alleviates salinity stress by improving cellular redox homeostasis, increasing photosynthesis, mitigating oxidative stress, and organizing the expression of stress-responsive genes [13]. The application of melatonin to exogenous or priming seeds can increase salinity alleviation in most plants [14].

Certain microorganisms belonging to different genera (e.g., *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Bacillus*) isolated from saline, alkaline, acidic, and arid soils can act as biostimulants. *Azotobacter chroococcum* can improve the stress tolerance of many plants, due to its nitrogen fixation abilities, and promote growth by producing phytohormones. Therefore, *Azotobacter chroococcum* is considered a plant growth-promoting rhizobacteria (PGPR) [15]. *Azotobacter chroococcum* can enhance root growth through expressing the ACC-deaminase, which reduces ethylene production in roots [16]. Under adverse conditions, exopolysaccharide (EPS)-producing bacteria have advanced strategies for adaptation and thriving. Among their modifications are an installation of the cell wall and the capability to assemble large quantities of solutes, which improve water detention and enhance the alleviation of osmotic and ionic stress. The structure of the cell wall is modified by EPS, which may form a protective biofilm on the root's surface [17].

The goal of these experiments was to evaluate the use of an integrated salinity stress management strategy including biostimulants (EPS-producing bacteria and melatonin) to ameliorate the adverse effects of salinity stress on faba bean plants, considering non-saline soil as a standard to assess the ability of these biostimulants to alleviate the adverse effects of salinity.

2. Materials and Methods

Field trials were conducted during the winter season of 2017/18 at two locations at Kafr El-Sheikh Governorate, Egypt. The first location included saline soil at Al Burullus (latitude: 31°35′56″ N; longitude: 31°21′ E; mean altitude: 5 m above sea level) and the second location included natural non-saline soil at Desouk (latitude: 31.14 N; longitude: 30.62 E; mean altitude: 6 m above sea level). The average annual temperature in Kafr El-Sheikh is 20.0 °C. The average annual rainfall is 72 mm. The temperatures are highest, on average, in July, at around 26.4 °C. The lowest average temperature in the year occurs in January, when it is around 12.5 °C. Prior to starting the experiments, soil samples were taken from the experimental sites and analyzed for their physical and chemical properties (Table 1), following the methods described by Chapman and Pratt [18].

Table 1. Physico-chemical properties analysis of non-saline and saline soils used for the field trials.

Properties	Particle Size Distribution			Soil Texture	pH 1:2.5	ECe dSm^{-1}	Soluble Cations (meq l^{-1})				Soluble Anions ($meql^{-1}$)		
	Sand (%)	Silt (%)	Clay (%)				Na^+	K^+	Ca^{++}	Mg^{++}	HCO_3^-	Cl^-	SO_4^-
Location 1	54.7	26.8	18.5	Sandy loam	7.76	6.5	38.5	5.2	15.4	6.3	5.9	37.1	22.4
Location 2	28.9	55.5	15.6	Silty loam	7.98	1.47	6.4	1.5	5.2	1.8	1.2	8.5	5.2

2.1. Bacterial Inocula, Preparation, and Inoculation Techniques

Rhizobium leguminosarum strains TAL 1399 and EPS-producing bacteria (*Azotobacter chroococcum*) were obtained from the microbial culture collection of the Agricultural Microbiology Dept. National Research Centre, Cairo, Egypt. The *Azotobacter chroococcum* used in the study was previously isolated from rhizoplane and rhizospheric soil fraction salt-affected soil and was selected based on the fact that it performed better at all EC levels from 4 to 12 dS m^{-1}; its ability to produce exopolysaccharides,

15g L^{-1} media; its intensive root colonization ability, 7.80×10^5 g^{-1} root dry weight of maize; and its ACC-deaminase activity, 442 nmol *a*-ketobutarate g^{-1} biomass h^{-1} [19].

Rhizobium leguminosarum and *Azotobacter chroococcum* were used as peat-based inoculant for faba bean. These strains were cultured in mannitol yeast extract medium and neutral broth, respectively, for 72 h under shaking (100 rpm) conditions at 28 ± 1 °C. An optical density of 0.5 mL of each culture measured at a wavelength of 600 nm was achieved by dilution to maintain a uniform cell density (10^7–10^8 cfu mL^{-1} for *Rhizobium* and 10^8 cfu mL^{-1} for *Azotobacter*) and was impregnated into sterilized peat. The inoculant was left for 30 days to obtain high numbers. *Rhizobium leguminosarum* inoculation was done by pelleting the seeds with a peat-based inoculant using Arabic gum in all treatments [20]. In the case of *Azotobacter chroococcum* treatments, a mixture of *Rhizobium* and *Azotobacter* was impregnated into sterilized peat (1:1 *v/v*).

2.2. Melatonin Application

Melatonin (M5250 SIGMA Melatonin powder, 98% Germany) was dissolved in 100% ethanol at a concentration of 30 mM and stored at −20 °C. Faba bean seeds were primed with melatonin before planting by the stored solution diluted to 1 mM with 100% ethanol and then further diluted with water to 0, 25, 50, and 100 µM concentrations. Faba bean seeds were primed with 300 µL per 100-seed reagent and air dried at room temperature.

In field inoculation, faba bean seeds were primed with 300 µL per 100-seed reagent and air dried at room temperature. After that, seeds of faba bean were inoculated with 10 g of peat impregnated with *Rhizobium* or a mixture of *Rhizobium* and *Azotobacter* (bacteria inoculum to peat ratio of 1:1:1 w/w/w) mixed with 10% Arabic gum solution. This peat contained approximately 10^9 cells of g^{-1} peat *Rhizobium leguminosarum* and 10^8 cells of g^{-1} peat *Azotobacter chroococcum*.

2.3. Experimental Procedure

Faba bean (*Vicia faba* cv. Giza3) seeds were cleaned and primed in melatonin at 0, 25, 50, and 100 µM for 12 h and left to dry in open air. *Rhizobium leguminosarum* and *Az. chroococcum* were applied using peat-based inoculants, as described above. The experimental design was a randomized complete block design (RCBD) factorial with two factors. The first factor was bacterial inoculation (without and with) and the second factor was melatonin application (0, 25, 50, and 100 µM), which were put in a submain plot, and each treatment was triplicated. Two locations were selected to apply the previously treated saline (Burullus) and non-saline soil (Desouk). Each plot area was 10.5 m^2, three meters in length, and three and half meters in width. Each plot contained five ridges, seeds were planted on two sides of the ridge in the hills, the hills were spaced 20 cm apart, and three seeds were sown in each hill. The plants were thinned to one plant per hill at 21 days after sowing. Soil preparation, fertilizer application, and cultural operations followed the normal practices of bean cultivation in the vicinity.

2.4. Data Recording

For each plot, a total of 30 plants were fixed for each treatment (a random sample of ten plants was assigned for investigation in each plot, and a total of 30 plants were fixed for each treatment) to study the morphological characteristics at 75 days after sowing (vegetative growth). Vegetative growth characteristics (shoot height in cm, number of branches ($plant^{-1}$), and the fresh and dry weights of the shoots (g $plant^{-1}$)) were recorded during the vegetative stage. Photosynthetic pigments (chlorophyll a,b and carotenoids) were estimated using the method described by Arnon [21]. Proline was estimated according to Bates et al. [22], and nutrient concentrations were determined according to the methods described by Jackson [23]. The relative water content (RWC) was measured in the first fully expanded leaf (from the top) of randomly selected plants. The leaves were collected, sealed in plastic bags, and transported to the laboratory. After measuring the fresh weights, leaves were immersed in distilled water for 24 h in a refrigerator. Leaves were then placed over tissue paper for blotting, and the fully turgid weight was measured. Finally, the leaf samples were oven dried at 72 °C for 24 h, after

which the dry weight was recorded and calculated according to Cao et al. [24], based on the following Equation (1):

$$\text{RWC (\%)} = \text{(Fresh weight-Dry weight/Turgid weight-Dry weight)} \times 100. \quad (1)$$

In order to determine the mean values of the yield at harvest time and its related parameters, (i.e., seeds per plant (g), weight of 100 seeds (g), and seed yield (Kg ha^{-1})), a random sample of ten plants was assigned for investigation in each plot, and a total of 30 plants were fixed for each treatment.

2.5. Statistical Analyses

All measurements were conducted for three replications of each sample. The data were analyzed using randomized complete block design for factor A, with factor B being analyzed using a split plot in an analysis of variance (ANOVA) using MSTATC statistical software, and the values are reported as means ± standard deviation (*SD*). Treatment differences were compared using the Least Significant Difference (LSD) procedure at a 5% level of probability.

3. Results

3.1. Vegetative Growth Parameters of Faba Bean Plants

The results presented in Tables 2 and 3 show that all measured growth parameters of the faba bean plants grown in location (1) (saline soil) were lower than the parameters for those grown in location (2) (non-saline soil). The plants inoculated with EPS-producing bacteria (*Az. chroococcum*) showed a significant increase (at $p \leq 0.05$) compared to uninoculated plants for all growth parameters in both locations, irrespective of the melatonin treatment. The inoculation treatment increase in growth parameters (shoot length, leaf number, branch number, and shoot fresh and dry weight) compared to uninoculated treatment was 26%, 26%, 27%, 21%, and 23% in location (1) (Table 2), respectively, whereas this increase was 25%, 22%, 22%, 11%, and 18% in location (2), respectively (Table 3).

On the other hand, melatonin at different concentrations was tested to improve faba bean growth in location (1) and location (2) (Tables 2 and 3). The obtained results showed a significant increase (at $p \leq 0.05$) for all tested growth traits at all melatonin levels in both locations relative to the respective control plants, irrespective of the inoculation or uninoculated treatments. The stressed (location (1)) and non-stressed (location (2)) seeds primed with 100 µM melatonin showed the highest values in their growth parameters, with a significant increase over untreated or other tested concentrations in terms of the leaf number, branch number, and shoot fresh and dry weights, while the shoot length showed no significant difference between the 50 and 100 µM melatonin concentrations in both soils. The application of 100 µM melatonin, regardless of the inoculation treatment, improved the shoot length, leaf number, branch number, shoot fresh weight, and shoot dry weight by 45%, 69%, 71%, 64%, and 63%, respectively, over the respective controls in saline soil (Table 2). However, in non-saline soil, 100 µM melatonin improved the shoot length (20%), leaf number (43%), branch number (63%), shoot fresh weight (36%), and shoot dry weight (57%) over the respective controls (Table 3).

The data on the interaction between inoculation treatments and melatonin levels showed that the maximum increase for all tested growth traits was observed in the faba bean seeds primed with 100 µM melatonin and inoculated with EPS-producing bacteria in combination, compared to those tested alone in both locations (Tables 2 and 3).

Table 2. Effect of exopolysaccharide (EPS)-producing bacteria and melatonin on growth parameters of faba bean grown in location one (saline soil).

Treatments	Melatonin (μM)	Shoot Length (cm)	No of Leaves Plant^{-1}	No of Branches Plant^{-1}	Shoot FW (g Plant^{-1})	Shoot DW (g Plant^{-1})
Uninoculated	0	* 39.70 ± 0.6e	12.77 ± 0.70f	1.33 ± 0.21e	35.85 ± 0.9e	7.65 ± 0.06e
	25	42.00 ± 1.0e	17.50 ± 0.29e	1.73 ± 0.61d	42.28 ± 0.45d	9.46 ± 0.04d
	50	49.20 ± 1.7cd	19.95 ± 0.37d	1.98 ± 0.03cd	50.41 ± 0.66c	10.43 ± 0.06d
	100	56.00 ± 2.5b	24.65 ± 0.39bc	2.28 ± 0.41bc	61.47 ± 0.83b	13.65 ± 0.05b
	Means	** 46.68B	18.72B	1.83B	47.50B	10.30B
Inoculated	0	46.70 ± 0.8d	18.24 ± 0.15de	1.75 ± 0.63d	44.90 ± 0.64d	10.37 ± 0.18d
	25	51.70 ± 1.4c	22.88 ± 013c	1.93 ± 0.48cd	53.56 ± 0.52c	11.92 ± 0.03c
	50	67.30 ± 2.8a	25.19 ± 0.18b	2.60 ± 0.07b	60.37 ± 0.16b	12.87 ± 0.06bc
	100	69.00 ± 1.9a	27.82 ± 0.10a	2.98 ± 0.40a	70.80 ± 0.71a	15.57 ± 0.23a
	Means	58.68A	23.54A	2.32A	57.41A	12.66A

For each column, * values followed by the same small letters are not significantly different according to an Least Significant Difference (LSD) test at $p \leq 0.05$. ** Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3). FW = fresh weight, DW = dry weight.

Table 3. Effect of EPS-producing bacteria and melatonin on growth parameters of faba bean grown in location two (non-saline soil).

Treatments	Melatonin (μM)	Shoot Length (cm)	No of Leaves Plant^{-1}	No of Branches Plant^{-1}	Shoot FW (g Plant^{-1})	Shoot DW (g Plant^{-1})
Uninoculated	0	* 50.03 ± 0.99j	19.30 ± 0.30e	1.75 ± 0.1f	48.42 ± 3.1e	9.68 ± 0.2e
	25	53.10 ± 0.50f	22.00 ± 0.50d	2.05 ± 0.2e	53.18 ± 6.2d	11.72 ± 0.6d
	50	56.23 ± 0.38e	26.00 ± 0.50c	2.62 ± 0.1d	59.45 ± 8.1c	12.72 ± 0.9d
	100	57.83 ± 0.71e	28.70 ± 0.80b	3.05 ± 0.2bc	66.80 ± 7.5b	16.32 ± 0.8b
	Means	** 54.30B	24.00B	2.37B	56.96B	12.61B
Inoculated	0	60.06 ± 0.78d	24.70 ± 0.40c	2.20 ± 0.1e	55.16 ± 3.3d	12.28 ± 0.3d
	25	64.50 ± 0.38c	28.70 ± 0.60b	2.80 ± 0.2cd	59.90 ± 6.1c	14.08 ± 0.6c
	50	72.56 ± 0.67b	30.00 ± 0.80b	3.10 ± 0.1b	64.37 ± 6.8b	15.24 ± 0.7bc
	100	74.76 ± 0.57a	34.00 ± 0.50a	3.40 ± 0.2a	73.71 ± 8.4a	18.08 ± 0.6a
	Means	67.97A	29.35A	2.88A	63.29A	14.92A

For each column, * values followed by the same small letters are not significantly different according to an LSD test ($p \leq 0.05$). ** Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3). FW = fresh weight, DW = dry weight.

3.2. Yield and the Components of Faba Bean Plants

The data in Tables 4 and 5 was used to evaluate the crop yield in location (1) and location (2). Generally, the lowest averages for the 100-seed weight, seed weight (plant^{-1}), and seed yield (ha^{-1}) were recorded as 62.3 g, 39.8 g plant^{-1}, and 3.35 ton ha^{-1}, respectively, in the saline soil (location 1), while the plants grown in non-saline soil (location 2) displayed values of 74.42 g, 49.40 g plant^{-1}, and 4.58 ton ha^{-1}, respectively, for the same parameters. The results in Tables 4 and 5 showed that inoculation with *Az. chroococcum* significantly increased (at $p \leq 0.05$) the yield and its components compared to the uninoculated plants in both saline and non-saline soils, irrespective of the melatonin treatment. The magnitude of the increase in the average 100-seed weight, seed yield (plant^{-1}), and seed yield (ha^{-1}) resulting from EPS-producing bacteria inoculation was 27%, 19%, and 33%, respectively, in saline soil (Table 4), and 13%, 18%, and 12%, respectively, in non-saline soil (Table 5), compared to the uninoculated plants.

Plants treated with melatonin at different concentrations showed a greater 100-seed weight, seed weight (plant^{-1}), and seed yield (ha^{-1}) than the control (0 μM) plants in both locations, regardless of the inoculation treatments. A melatonin level of 100 μM was the most effective in increasing the above yield and its components significantly (at $p \leq 0.05$), compared to the other concentrations, except for the 100-seed weight, which had no significant effect in saline soil (Table 4). However, in non-saline soil, the increases in 100-seed weight, seed weight (plant^{-1}), and seed yield were not significant between

50 and 100 µM melatonin concentrations (Table 5). The application of 100 µM melatonin improved, on average, the 100-seed weight by 56% and 44%, the seed weight (plant^{-1}) by 56% and 53%, and the seed yield (ha^{-1}) by 42% and 28% in saline and non-saline soils, respectively, compared to the respective control plants.

Table 4. Effect of EPS-producing bacteria and melatonin on yield components of faba bean grown in location one (saline soil).

Treatments	Melatonin (µM)	Average 100-Seed Weight (g)	Seed Weight (g Plant^{-1})	Seed Yield (Ton ha^{-1})
Uninoculated	0	* 41.6 ± 0.05j	26.19 ± 1.9e	2.20 ± 0.03j
	25	54.63 ± 0.05e	34.80 ± 1.3d	2.69 ± 0.05f
	50	60.63 ± 0.06d	39.20 ± 1.7c	3.10 ± 0.06e
	100	62.97 ± 0.07c	45.10 ± 2.3b	3.47 ± 0.03cd
	Means	** 54.96B	36.32B	2.87B
Inoculated	0	48.69 ± 0.06f	35.17 ± 1.5d	3.26 ± 0.06de
	25	73.83 ± 0.07b	40.70 ± 1.2c	3.75 ± 0.05bc
	50	77.73 ± 0.07a	46.60 ± 1.3b	4.01 ± 0.06b
	100	78.30 ± 0.04a	50.62 ± 1.5a	4.30 ± 0.07a
	Means	69.64A	43.27A	3.83A

For each column, * values followed by the same small letters are not significantly different according to an LSD test at $p \leq 0.05$. ** Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3).

Table 5. Effect of EPS-producing bacteria and melatonin on yield components of faba bean grown in location two (non-saline soil).

Treatments	Melatonin (µM)	Average 100-Seed Weight (g)	Seed Weight (g Plant^{-1})	Seed Yield (Ton ha^{-1})
Uninoculated	0	* 53.43 ± 0.5j	33.49 ± 1.5f	3.70 ± 0.05f
	25	68.53 ± 0.5e	42.21 ± 1.9e	4.37 ± 0.05e
	50	76.37 ± 0.7d	50.07±1.4d	4.77 ± 0.06c
	100	81.27 ± 0.6c	55.57 ± 1.3c	4.87 ± 0.05c
	Means	** 69.90B	45.30B	4.31B
Inoculated	0	64.37 ± 0.04f	41.70 ± 1.7e	4.32 ± 0.06e
	25	78.87 ± 0.05c	55.42 ± 1.5c	4.57 ± 0.09d
	50	83.73 ± 0.06b	57.70 ± 2.3b	5.10 ± 0.08b
	100	88.73 ± 0.05a	59.15 ± 1.4a	5.38 ± 0.09a
	Means	78.925A	53.49A	4.84A

For each column, * values followed by the same small letters are not significantly different according to an LSD test at $p \leq 0.05$. **Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3).

The interaction effect of the inoculation treatments and melatonin concentrations, and the average 100-seed weight, seed yield (plant^{-1}), and seed yield ha^{-1} of plants inoculated with EPS-producing bacteria and primed with 100 µM melatonin in combination recorded the highest values in both locations. On the other hand, there were no significant (at $p \leq 0.05$) differences between (50 and 100 µM) melatonin concentrations when combined with EPS-producing bacteria inoculation for all studied yield components in both locations. There were no significant (at $p \leq 0.05$) differences between the plants treated with 50 µM melatonin combined with EPS-producing bacteria and the plants treated with 100 µM melatonin alone without inoculation in non-saline soil, for all studied yield components.

3.3. Effects of Melatonin and EPS-Producing Bacteria on the Physiological Attributes of Faba Bean Plants

The results presented in Tables 6 and 7 were employed to evaluate the effects of EPS-producing bacteria and melatonin on the photosynthetic pigments, RWC%, and proline content of faba bean

plants grown in location (1) and location (2). The results indicate that salinity stress causes a reduction in chlorophyll a,b, carotenoids, and RWC, while the proline content is increased.

Table 6. Effect of EPS-producing bacteria and melatonin on photosynthetic pigments, relative water content (RWC)%, and proline content of faba bean plants grown in location one (saline soil).

Treatments	Melatonin (µM)	Photosynthetic Pigments (mg g^{-1} FW)			RWC (%)	Proline (mg g^{-1} FW)
		Chlorophyll *a*	Chlorophyll *b*	Carotenoid		
Uninoculated	0	* 0.809 ± 0.430e	0.283 ± 0.025j	0.171 ± 0.014k	45.32 ± 0.19j	0.251 ± 0.07f
	25	0.936 ± 0.025d	0.315 ± 0.020e	0.187 ± 0.015j	55.79 ± 0.15f	0.293 ± 0.06ef
	50	1.049 ± 0.025cd	0.356 ± 0.024d	0.202 ± 0.014f	60.37 ± 0.19d	0.327 ± 0.06de
	100	1.162 ± 0.028bc	0.509 ± 0.008b	0.230 ± 0.021d	65.79 ± 0.23c	0.348 ± 0.07cd
	Means	** 0.989B	0.366B	0.198B	56.82B	0.305B
Inoculated	0	1.056 ± 0.023cd	0.383 ± 0.022d	0.219 ± 0.022e	58.00 ± 0.17e	0.362 ± 0.07cd
	25	1.147 ± 0.017bc	0.451 ± 0.026c	0.245 ± 0.020c	65.00 ± 0.23c	0.395 ± 0.07c
	50	1.258 ± 0.066b	0.585 ± 0.023a	0.284 ± 0.018b	70.23 ± 0.18b	0.474 ± 0.08b
	100	1.385 ± 0.020a	0.616 ± 0.022a	0.292 ± 0.015a	75.80 ± 0.24a	0.526 ± 0.10a
	Means	1.212A	0.509A	0.260A	67.26A	0.439A

For each column, * values followed by the same small letters are not significantly different according to an LSD test at $p \leq 0.05$. ** Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3). FW = Fresh weight, RWC = Relative water content.

Table 7. Effect of EPS-producing bacteria and melatonin on photosynthetic pigments, RWC%, and proline content of faba bean plants grown in location two (non-saline soil).

Treatments	Melatonin (µM)	Photosynthetic Pigments (mg g^{-1} FW)			RWC (%)	Proline (mg g^{-1} FW)
		Chlorophyll *a*	Chlorophyll *b*	Carotenoid		
Uninoculated	0	* 1.155 ± 0.014f	0.405 ± 0.014e	0.235 ± 0.007e	59.35 ± 0.94f	0.110 ± 0.01d
	25	1.253 ± 0.046e	0.445 ± 0.006d	0.245 ± 0.014e	67.75 ± 0.44e	0.115 ± 0.07d
	50	1.286 ± 0.020e	0.503 ± 0.006c	0.284 ± 0.023d	70.76 ± 0.48d	0.122 ± 0.05d
	100	1.340 ± 0.009d	0.544 ± 0.020b	0.292 ± 0.031cd	74.77 ± 0.45c	0.159 ± 0.07c
	Means	** 1.259B	0.474B	0.264B	68.160B	0.127B
Inoculated	0	1.345 ± 0.028d	0.504 ± 0.029c	0.284 ± 0.029d	66.6 ± 0.12e	0.150 ± 0.08c
	25	1.418 ± 0.051c	0.525 ± 0.023bc	0.305 ± 0.040bc	74.01 ± 0.12c	0.213 ± 0.06b
	50	1.456 ± 0.014b	0.574 ± 0.009a	0.312 ± 0.011b	80.82 ± 0.039b	0.231 ± 0.09b
	100	1.499 ± 0.028a	0.603 ± 0.011a	0.329 ± 0.008a	84.46 ± 0.31a	0.263 ± 0.07a
	Means	1.430A	0.552A	0.308A	76.47A	0.214A

For each column, * values followed by the same small letters are not significantly different according to an LSD test at $p \leq 0.05$. ** Means of inoculation treatments followed by the same capital letters are not significantly different according to an LSD test at $p \leq 0.05$. Data are displayed as means ± standard deviation (n = 3). FW = Fresh weight, RWC = Relative water content.

The data in Tables 6 and 7 show that inoculation with EPS-producing bacteria, irrespective of the melatonin treatment, mitigated the adverse salt stress effects by significantly increasing (at $p \leq 0.05$) photosynthetic pigments (chlorophyll a,b and carotenoid content). The magnitude of the increase in chlorophyll a,b and carotenoid content was 22%, 39%, and 33%, respectively, compared to the corresponding uninoculated beans in location (1) (salt stress) (Table 6), and increased by 14%, 16%, and 17%, respectively, compared to the corresponding uninoculated beans in location (2) (Table 7). Similarly, bacterial inoculation significantly (at $p \leq 0.05$) increased the RWC% and proline content by 18% and 44%, respectively, in saline soil, and by 12% and 69%, respectively, in non-saline soil, compared to the respective uninoculated controls.

As shown in Tables 6 and 7, regardless of the inoculation treatment, seeds primed with melatonin significantly (at $p \leq 0.05$) improved all fractions of photosynthetic pigments, RWC%, and proline content under all three melatonin concentrations compared to the respective controls in both locations. Increasing the melatonin levels significantly (at $p \leq 0.05$) increased chlorophyll a,b, carotenoids, and RWC, regardless of the inoculation treatment, in saline soil, while in non-saline soil, there were no significant differences between 50 and 100 µM melatonin concentrations in chlorophyll a,b, carotenoids,

and RWC. Increasing the melatonin levels significantly increased the proline content in both soils. Treatment with the 100 µM melatonin concentration resulted in the highest increase of chlorophyll a,b, carotenoids, RWC, and proline content, with values of 36%, 69%, 34%, 37%, and 42% in saline soil and 14%, 26%, 20%, 26%, and 62% in non-saline soil, for the same parameters, over the respective control.

Generally, for the interaction effect, it was noted that dual treatments (inoculation with EPS-producing bacteria and seed priming with melatonin) not only mitigated the inhibitory effects of salt stress, but also enhanced the stimulating effect compared to the control and single-treatment plants in both saline and non-saline soils (Tables 6 and 7). The maximum values of chlorophyll a,b, carotenoids, RWC, and proline content were observed in the combination of EPS-producing bacteria inoculation and priming with 100 µM melatonin in both locations.

3.4. Effect of Melatonin and EPS-Producing Bacteria on the Nutrient Concentrations of Faba Bean Plants

Figures 1 and 2 show that the concentrations of N, P, and K decreased when increasing the concentrations of Na^+ and Cl^-, which adversely affected the K^+/Na^+ ratio in faba bean plants grown in saline soil compared to non-saline soil. EPS-producing bacteria inoculation, irrespective of the melatonin level, provided significant increases (at $p \leq 0.05$) in N, P, and K concentrations and consistently significantly decreased the concentrations of Na^+ and Cl^- in the plant tissues grown in both studied soils compared to the uninoculated plants (Figures 1 and 2). Inoculated plants with EPS-producing bacteria are clearly superior in terms of absorbing beneficial nutrients and excluding harmful ones.

Regardless of the inoculation treatment, plants applied with melatonin at all three concentrations increased N, P, and K concentrations and decreased concentrations of Na^+ and Cl^- significantly (at $p \leq 0.05$) compared to the untreated plants. Seeds primed with melatonin significantly increased (at $p \leq 0.05$) the K^+/Na^+ ratio compared to the untreated plants in both saline and non-saline soils. The increase in N, P, and K concentrations and the K^+/Na^+ ratio, and the reduction in Na^+ and Cl^- concentrations, resulting from increasing the melatonin levels, were significant (at $p \leq 0.05$) in both soils. Furthermore, 100 µM melatonin was found to be more effective than other concentrations in suppressing salt stress. However, the N and P concentrations in plants treated with 50 and 100 µM melatonin differed significantly (at $p \leq 0.05$) in saline soil only (Figures 1 and 2). For inoculated seeds with EPS-producing bacteria, those primed with 100 µM melatonin in combination achieved the highest N, P, and K concentrations and K^+/Na^+ ratios. In contrast, the plants treated with a combined treatment produced the lowest Na^+ and Cl^- concentrations in both soils.

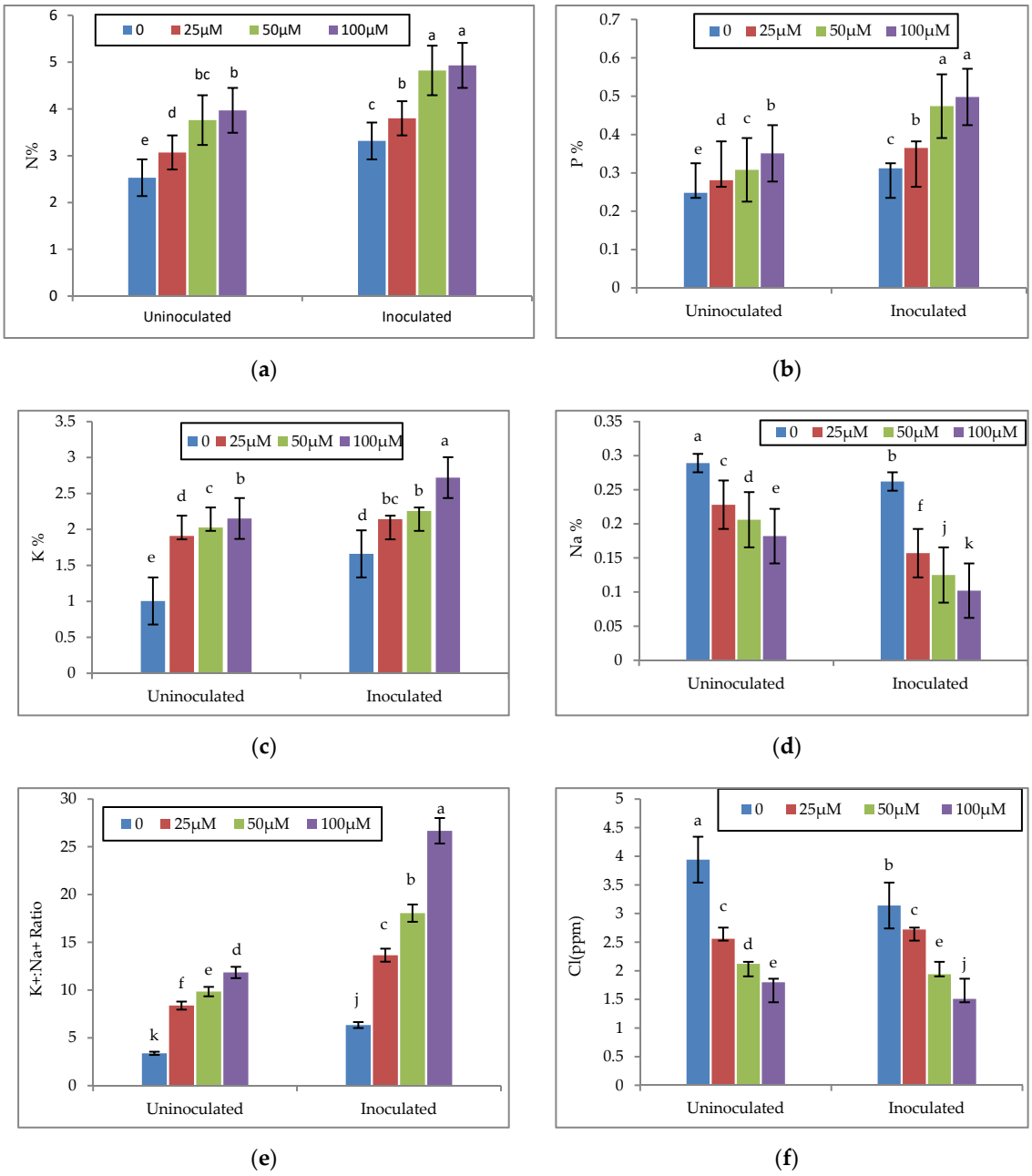

Figure 1. Effect of EPS-producing bacteria and melatonin on nutrient concentrations [N% (**a**), P% (**b**), K% (**c**), Na% (**d**) and Cl ppm (**f**)] and the K^+/Na^+ ratio (**e**) of faba bean grown in location one (saline soil). The bars of treatment followed by the same letter were not statistically different at $p \leq 0.05$, according to the least significance difference (0, 25, 50, and 100 µM concentrations of melatonin).

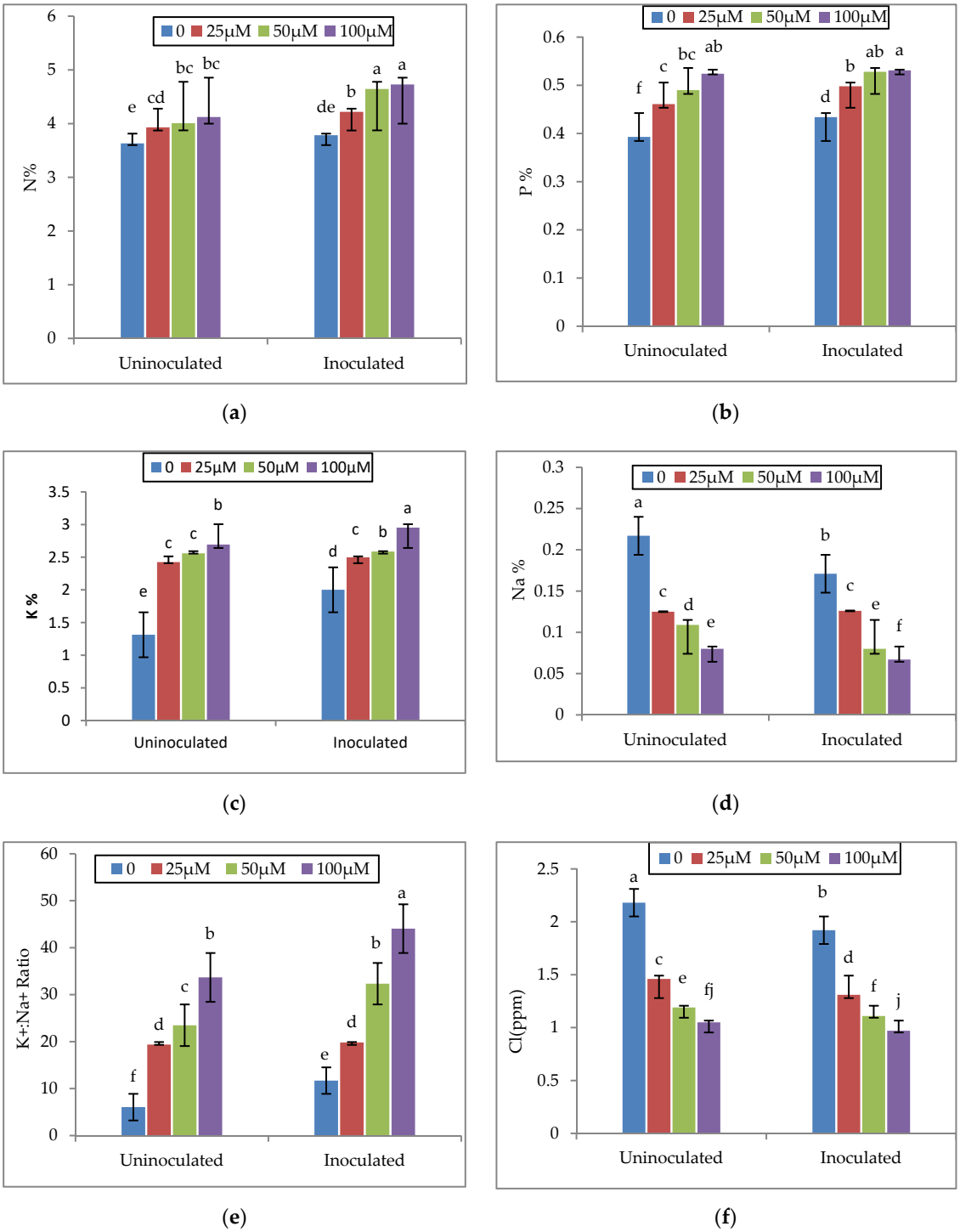

Figure 2. Effect of EPS-producing bacteria and melatonin on nutrient concentrations [N% (**a**), P% (**b**), K% (**c**), Na% (**d**) and Cl ppm (**f**)] and the K^+/Na^+ ratio (**e**) of faba bean grown in location two (non-saline soil). The bars of treatment followed by the same letter were not statistically different at $p \leq 0.05$, according to the least significance difference (0, 25, 50, and 100 µM concentrations of melatonin).

4. Discussion

In arid and semiarid areas, agricultural productivity suffers from great problems due to salinity, which are caused by several factors [25]. In these field studies, seeds of faba bean inoculated with EPS bacteria and primed with melatonin alone or in combination were evaluated for their effects on

the growth, physiological attributes, nutrient status, and yield of faba bean grown in two locations differing in terms of salinity: saline soil (location (1), ECe = 6.5 dSm^{-1}) and non-saline soil (location (2), ECe = 1.47 dSm^{-1}). The present study showed that salinity stress reduced the growth and yield of faba bean plants. This result was clearly observed in saline soil (Tables 2 and 4) compared to non-saline soil (Tables 3 and 5). These results run parallel to the results obtained by Fathalla [7], Castanares, and Bouzo [14]. The reduction in the growth and yield could be attributed to preventing cell division, cell magnification, and extension [26], or due to osmotic stress and ion toxicity [27], under salinity stress. NaCl affects the permeability of the plasma membrane and increases the influx of external ions (Na^+) and the efflux of cytosolic solutes in plant cells [28]; it also causes hardening of the cell wall [29].

Inoculation with EPS-producing bacteria promotes plant growth in both saline and non-saline soils. This agrees with Arora et al. [30], who stated that EPS-producing bacteria promote plant growth by releasing phytohormones, which boost bacterial root colonization and biofilm formation. Inoculation with *Az. chroococcum* has been shown to increase the growth and yields of wheat, cotton, pea, potato, rice, and tomato under salinity stress [31–33]. Fathalla [7] and Velmourougane et al. [17] showed that biofilm formation and EPS-production by *Az. chroococcum* significantly contribute to soil fertility and improve plant growth. Wheat plants inoculated with salt-tolerant *Azotobacter* strains increased their biomass, nitrogen content, and grain yield under salt stress [34]. The obtained results indicate that the beneficial effects of inoculation are more pronounced for the average 100-seed weight and seed yield (ha^{-1}) in plants grown in saline soil than those grown in non-saline soil. Similarly, Ashraf et al. [35] concluded that the inoculation of EPS-producing bacteria is a valuable tool for ameliorating and increasing crop productivity under salinity stress. In this respect, Qurashi and Sabri [36] reported that inoculation with EPS-producing bacteria at a higher salt stress up to 100 mM increased soil aggregation, which was more pronounced around roots, while aggregates formed by exopolysaccharides favored plant growth under salt stress.

Wei et al. [37] noted that melatonin may improve the growth and seed yield under both normal and salt stress conditions by activating photosynthesis, the metabolism of carbohydrates and antioxidative actions, DNA replication, and cell division. The obtained results indicate that the beneficial effects of melatonin on plant growth are more pronounced in plants grown in saline soil than those grown in non-saline soil. In this respect, melatonin may act as an auxin, an indole acetic acid (IAA) [38], or an antioxidant [39], to resist biotic/abiotic stress, which has been observed with different plant species [14,40].

The results also show that reductions in chlorophyll a,b and carotenoids are caused by salinity stress. Similar results have been observed by Castanares and Bouzo [14], Mahmood et al. [41], and Li et al. [42]. Salinity stress causes chlorophyll degradation and/or damages the photosynthetic apparatus by forming proteolytic enzymes, such as chlorophyllase, resulting in a decreased chlorophyll content [25]. Hahm et al. [43] also reported that reducing RWC is an ideal action for helping plants to resist osmotic stress. The accumulation of proline under salinity stress is considered to be an indicator in several plant species [14,44]. This compound acts in the protection of cellular components by dehydration, maintaining the membrane structure and acting as a free radical scavenger [45].

Bacterial inoculation inhibits chlorophyll degradation by increasing the ACC-deaminase enzymes in plant growth-promoting rhizobacteria (PGPR)-inoculated plants, which increase the photosynthetic rate [46]. The effective uptake of nutrients may be due to the role of nitrogen nutrition in producing growth-promoting substances. A similar result was observed for mung bean [41] and soybean [47]. In this respect, Hahm et al. [43] noted that the RWC and proline contents of the non-inoculated control plants were lower than those of the plants inoculated under both saline and normal conditions. There are several strategies that EPS-producing bacteria-treated plants use to adapt under stress conditions [48]; among these strategies are the production and/or uptake of osmolytes that may have contributed to maintaining favorable water uptake [33]. In addition, EPS-producing bacteria may help in maintaining a film of hydration around the roots and/or help re-establish favorable water potential gradients under water limitations [49]. Hahm et al. [43] reported that the increased proline content

in pepper plants inoculated with PGPR may be due to PGPR strains enhancing metabolic defense strategies under saline conditions.

Similarly, the stimulatory effect of melatonin on the physiological attributes of faba bean plants was much more pronounced under salinity stress. This result indicates an improved adaptation to saline environments. Han et al. [50] and Siddiqui et al. [51] noted that exogenous melatonin significantly prevents chlorophyll degradation under abiotic stress, which maintains the photosynthetic capacity in saline soils. The inhibition effect of chlorophyll degradation by melatonin is likely due to an increase in antioxidant enzyme activities and antioxidant content, thus inhibiting the production of reactive oxygen species [52]. Castanares and Bouzo [14] reported that increasing the RWC under melatonin treatment resulted in a better water absorption capacity. Siddiqui et al. [51] reported that the proline content increased in salt-affected plants and in melatonin-treated plants. This could be attributed to the antioxidation effect of melatonin, which inhibits proline degradation [53]. Many mechanisms consider that melatonin protects plants against salinity stress.

Under stress conditions, the availability of nutrients in the soil is reduced due to a decreased solubility, which leads to decreased absorption and concentrations in plants [54]. The results observed in the present study for nutrient concentrations are parallel to the results obtained by Gomes et al. [55], who reported that the Na^+, Cl^-, and Na/K ratios increased, while Ca^{2+}, K^+, and Mg^{2+} reduced with an increasing salinity; N and P showed no significant differences among saline treatments. Castanares and Bouzo [14] reported that the K concentration decreased and the Na^+/K^+ ratio increased under salinity stress. Excessive Na^+ and Cl^- concentrations affected the absorption of many essential nutrients, such as K, Ca, Mg, and N [56,57]. This could be attributed to the competitive interactions affecting the ionic selectivity of cell membranes [58] and photosynthetic activity [59]. The increase in Na^+ concentrations and decrease in K^+ concentrations and K^+/Na^+ ratios in plant leaves is characteristic of Na^--induced toxicity [60], which can be attributed to the effects of competition between the identically-charged ions of Na^+ and K^+ during nutrient absorption at the absorptive sites of plant roots [61]. A reduction in the K^+ concentration (Figure 1) could inhibit growth (Table 2) by reducing the capacity for osmotic adjustment and turgor maintenance or by adversely affecting metabolic functions (Table 6) [62].

Inoculation with *Azotobacter* strains was shown to increase phosphorous and nitrogen availability [63] and, therefore, facilitate the uptake of N, P, and K and the exclusion of Na^+ under saline stress. The EPS molecules produced by bacteria have a charged part that reacts with, and chelates, sodium ions [30]. Inoculated plants with PGPR are clearly superior in terms of absorbing beneficial nutrients and excluding harmful ones, likely to maintain the nutrient balance [46]. Similar to the obtained results, Li et al. [64] reported that, under salinity, melatonin might control the expression of ion-channel genes that enhance salinity tolerance.

An integrated salinity stress management strategy integrating the external application of melatonin and EPS-producing bacteria (*Az. chroococcum*) can lead to continuous and effective agricultural practices that will manage the stress and boost yield of faba bean plants. The results revealed that the best response occurs in seeds of faba bean inoculated with EPS-producing bacteria and primed with melatonin in combination. These plants more successfully increased the growth and yield of faba beans, photosynthetic pigments, proline content, and RWC%, and increased the N, P, and K concentrations, but showed declined concentrations of Na^+ and Cl^-, which led to a higher ratio of K^+/Na^+ compared to single treatments (EPS bacteria or melatonin application alone) under both salt stress and unstressed conditions. This effect is likely due to the additive effects of EPS-producing bacteria and melatonin application. In the current study, the addition of melatonin and inoculation with EPS-producing bacteria complemented each other to improve the vegetative growth parameters and yield of faba bean under salinity conditions. It may be possible that melatonin was utilized by the soil bacteria to sustain natural microbial activity by coping with the impacts specific to salt stress, such as an enhanced osmotic pressure and ion toxicity [65,66]. Other reports have also suggested that melatonin may enhance abiotic stress tolerance in microbes, as endogenous levels of melatonin increased under abiotic stress [67,68].

5. Conclusions

This study quantified the effects of salinity on the growth and yield of faba bean plants. EPS-producing bacteria inoculation and melatonin application were assessed individually or in combination for their ability to alleviate the adverse effects of salinity. This study has shown that both melatonin and EPS-producing bacteria may be efficient act as bio-stimulants to improve the tolerance of faba bean plants to salinity. On the other hand, priming seeds in melatonin and inoculating them with EPS-producing bacteria was the most effective combination for improving the growth and seed yield of faba beans, photosynthetic pigments, and water relationships in saline soil. In addition, the combined use of melatonin and EPS-producing bacteria has been shown to have a stimulating effect, which may represent an evolving sustainability strategy to mitigate salinity stress in faba bean plants. Therefore, further studies are needed to test whether melatonin and EPS-producing bacteria can be used as a general strategy to improve the stress tolerance of other crops.

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