Journal of the Saudi Society of Agricultural Sciences (2025) 24:1
https://doi.org/10.1007/s44447-025-00002-1

ORIGINAL PAPER

Effects of melatonin and bacterial bio-stimulants on calendula officinalis under salinity stress

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Received: 8 January 2025 / Accepted: 1 March 2025 / Published online: 24 March 2025
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Abstract

Melatonin can mitigate the adverse effects of salt stress in plants, but the combined effects of it and bacterial bio-stimulants on enhancing plant resilience to salinity remain poorly understood. To fill in such a knowledge gap, we investigated the interaction between melatonin and bacterial bio-stimulants on the growth, productivity, and physiological characteristics of *Calendula officinalis* seedlings under salt stress. Using a pot-sand culture method, we tested various concentrations of exogenous melatonin (distilled water, 50, 100 µM) combined with a bacterial mixture (5 ml/pot at 10^8 CFU/ml) of *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens*. The seedlings were subjected to different saline irrigation levels with NaCl (tap water, 42.8, 85.6, and 128.3 mM) over two growing seasons in 2023 and 2024. The experiment was designed as a factorial study using a randomized complete block design with three replicates. NaCl concentrations of 85.6 mM and 128.3 mM negatively affected seedling growth and yield. Foliar application of 100 µM melatonin combined with bacterial inoculation significantly enhanced leaf area and shoot dry weight. Inflorescence parameters improved notably, with increases in total carotenoids and flavonoids in petals. Total chlorophyll content, mineral percentages (N, P, K), and proline increased significantly, while sodium and chloride levels decreased. The activities of polyphenol oxidase and peroxidase increased significantly and also improved relative water content and reduced electrolyte leakage. Our findings suggest that the combination of exogenous melatonin and bacterial inoculation can effectively mitigate the adverse effects of salt stress on Calendula seedlings.

Keywords Saline irrigation · Bacterial-inoculation · Marigold · Carotenoids · Flavonoids

1 Introduction

Abiotic stresses, which are increasingly exacerbated by climate change, significantly impede plant growth and development, thereby adversely affecting crop yield and quality. This situation poses a substantial threat to the sustainability of global agricultural production systems (Yadav et al 2020). Among these abiotic stresses, salinity is particularly detrimental, leading to marked reductions in crop growth and overall productivity (Wang and Huang 2019). The mechanisms underlying salinity stress include osmotic stress, ionic toxicity, and nutrient imbalances, which collectively result in the excessive production of reactive oxygen species (ROS) (Hossain and Dietz 2016). In Egypt, climate change directly impacts water availability and facilitates saltwater intrusion into groundwater, thereby increasing agricultural vulnerability. (Omar et al 2021). Thus, sustainable practices are urgently needed to enhance yields and rehabilitate saline soils.

Extracts from pot marigold (*Calendula officinalis*) exhibit a diverse array of pharmacological properties, such as antiseptic, stimulant, diaphoretic, antispasmodic, and antipyretic effects (Ukiya et al 2006). These extracts have been utilized for internal treatments of conditions such as gastritis, colitis, and bleeding associated with duodenal ulcers (Preethi et al 2009). Furthermore, inflorescences are commonly prepared as extracts, tinctures, and balms for external applications to address skin inflammations, lacerations, and hemorrhagic wounds. The

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therapeutic potential of pot marigolds is attributed to their rich composition of bioactive compounds, which contribute to their efficacy in various medical applications (Patil et al 2022). The significant biological activity of *C. officinalis* and its constituents underscores the necessity for further research and development of this plant as a medicinal resource.

Recent studies have confirmed the protective roles of melatonin (Mel) (N-acetyl-5-methoxytryptamine) against various stress conditions in plants which indicates that the exogenous application of melatonin significantly enhances plant tolerance to abiotic stresses, including salinity (Khan et al 2022). In addition to its benefits for plants, melatonin concentrations in food products may positively influence human health due to its biological activities. It has been suggested that melatonin could mitigate adverse anatomical and physiological conditions related to aging, cancer, diabetes, and cardiovascular diseases (Iriti et al 2010). The multifaceted roles of Mel in enhancing stress tolerance and promoting overall plant health warrant further investigation into its potential applications in agricultural practices.

The application of bacterial bio-stimulants represents a sustainable and environmentally friendly strategy for mitigating salt stress in soils. This approach not only helps to rehabilitate saline soils but also enhances their nutritional quality (Mishra et al 2022). Specific PGPR strains have shown promise as bioinoculants to improve soil fertility and provide protection to plants under salt-stress conditions (Qin et al 2014). These bacteria operate through mechanisms like phytohormone production, enhanced nutrient acquisition, and improvements in soil structure (binding capacity, cation exchange, water retention, bulk density reduction) and the synthesis of exopolysaccharides, antioxidants, and osmolytes (Nadeem et al 2015). Notable genera of salt-tolerant PGPR include Azotobacter, Bacillus, and Pseudomonas (Arora et al 2020) whereas *Pseudomonas* spp. contribute to plant growth by reducing sodium ion uptake and increasing the activity of antioxidant enzymes in saline environments (Fu et al 2010). Furthermore, Bacillus strains such as *B. megaterium* are well-documented for their ability to promote plant growth under salinity stress (Wang et al 2018). Additionally, *Azotobacter chroococcum* has been reported to improve sunflower growth when exposed to salinity stress (Abdelaziz 2022). This evidence supports the potential of utilizing PGPR as a biological tool for enhancing plant resilience against salinity, thereby contributing to improved agricultural productivity in saline-prone regions.

Combining melatonin and PGPR has been demonstrated to be more efficacious than their individual use in enhancing plant growth and yield, and mitigating salinity toxicity under saline conditions (Yang et al 2020; Alinia et al. 2022). In light of the pressing need for sustainable strategies to counteract the detrimental effects of salinity on plants and soil, this study aimed to investigate the interaction between melatonin foliar spraying and bacterial inoculation on the growth and productivity of *Calendula officinalis* under saline irrigation. This research contributes to the development of innovative, eco-friendly approaches to improve plant resilience under salinity stress, aligning with the imperative to address the global challenges posed by soil salinization.

2 Materials and methods

In the two consecutive seasons of 2022–2023 and 2023–2024, an outdoor pot experiment was carried out in the experimental farm of the Faculty of Agriculture, Ain Shams University, Qalubia Governorate, Egypt. We purchased *Calendula officinalis* seeds from Fayoum (El-Hamzawy company). During both seasons, seeds were sown in the nursery on 10th October and transplanted after forty-five days from sowing into plastic pots (one plant/25 cm diameter pot) filled with approximately 7 kg of washed sand with the addition of 71 g of compost per pot. Table 1 presents the results of the chemical analysis of the compost used.

Mineral fertilization was added twice, the first addition was after 45 days from transplanting and the second was after 30 days from the first addition as follows: ammonium sulfate (20.5%N) at the rate of 400 kg/feddan (7.0 g/ pot), calcium superphosphate (15.5% P_2O_5) at the rate of 400 kg/fed (7.0 g / pot) and potassium sulfate (48% K_2O) at the rate of 150 kg/fed (2.7 g/ pot).

2.1 Treatments and experimental layout

The experiment consisted of three factors. The first factor was saline water irrigation, which had four levels: Tap water (S_0), 42.8 mM (S_1), 85.6 mM (S_2), and 128.3 mM (S_3). Highly soluble NaCl was used to prepare different levels of saline irrigation. In this experiment, plants received saline irrigation water in an amount that matched the field water capacity (63%) of the soil (Khalid and da Silva 2010).

The second factor was a foliar application with melatonin (Alfa Aesar, Kandel, Germany) with three levels: distilled water (M_0), 50 µM (M_1), and 100 µM (M_2). Plants were sprayed with Mel six times. The first one was after two weeks of transplanting and repeated every 15 days till ninety days after transplanting with the addition of Tween-20 as a wetting agent.

Table 1 Chemical analysis of the used compost

P^H	EC (ds/m)	Organic matter %	Organic carbon %	N%	P%	K%	C: N ratio
7.3	2.02	25.86	22.95	1.82	0.49	0.29	12.61

The third factor was the use of bio-stimulants by adding a bacterial mixture of *Azotobacter chroococcum* (10^8 CFU/ml.), *Bacillus megaterium* (10^8 CFU/ml.), *Pseudomonas fluorescens* (10^8 CFU/ml.) in two levels; the use (B_1) or non-use (B_0) of the bacterial mixture. These strains were mixed in equal amounts (1:1:1), and then 5 ml/pot was injected beside the plant into the wet soil in liquid form after two weeks of transplanting and after 30 days from the first addition. All inoculants were provided by the Bio-fertilizer Unit, Faculty of Agriculture, Ain Shams University, Egypt.

The experiment was carried out in a randomized complete blocks design (R.C.B.D) with three replicates. Each replicate contained four plants in twenty-four treatments (4*3*2) as shown below in Fig. 1 and 2. The number of experimental units was 288 pots (24*3*4).

2.2 Data recorded

2.2.1 Vegetative measurements

After 120 days from transplanting, plants were dried at 70^0 C to a constant weight to record the **dry weights of the herb (g/plant). Leaf area (cm^2)** was recorded after 90 days of transplanting according to Giuffrida et al (2011) using the following equation.

Leaf area = 0.53 + 0.56 LW

wherein: L = length and W = width.

2.2.2 Inflorescences parameters

Inflorescences were harvested immediately after the full opening throughout the flowering period then, a **diameter of inflorescence (cm)** was recorded. The inflorescences were air-dried in the shade to record their **dry weight (g/plant)**. **Inflorescences count /plant** was recorded by collecting the number of inflorescences per plant during the flowering period.

Determination of total carotenoids of petals Total carotenoids were estimated by Siebeneichler et al (2019). 0.1 g of dried petals were extracted in 10 ml of N, N-dimethylformamide (DMF) and kept for 48 h in the dark, then the pigment quantification was performed by spectrophotometry with wavelengths of 470 nm for carotenoids, 665 nm and 646 nm for chlorophylls.

$$Carotenoids = \frac{1000 * A470 - 3.27Chl\ a - 104 * Chl\ b}{198}$$

wherein: A = wavelengths.

Determination of flavonoids of petals To measure the total flavonoid content, the aluminum chloride colorimetric method was used, following the guidelines set by Chang et al (2002)**.** Initially, 0.5 g of dried petals was extracted in methanol. Subsequently, 0.5 ml of the extract was combined with 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The resulting

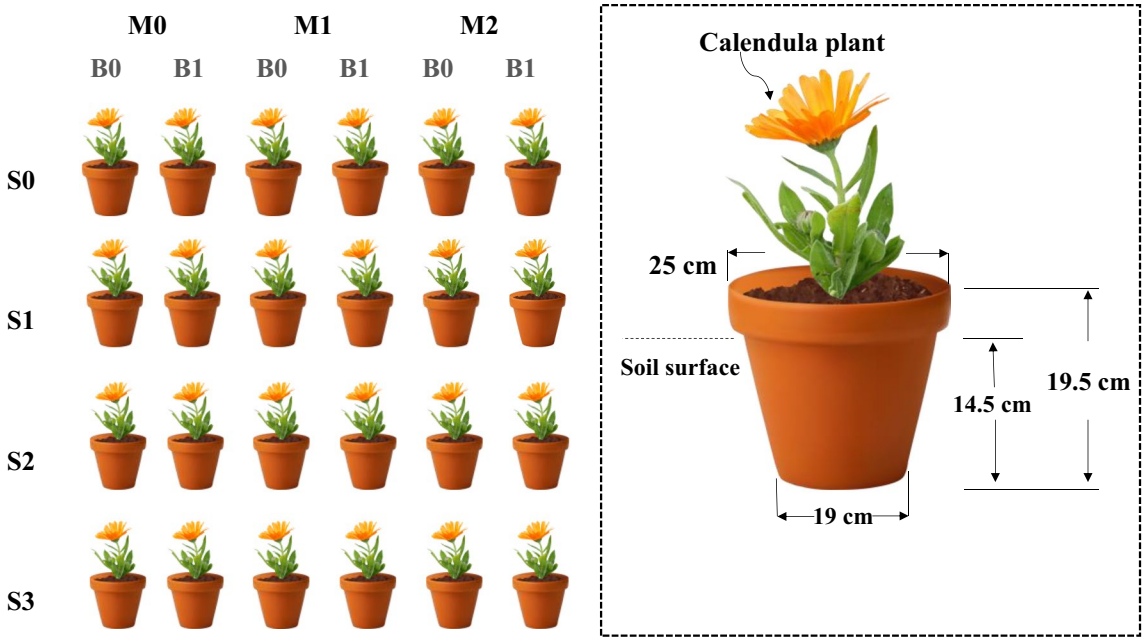


Fig. 1 A layout of the experiment. Salinity levels (S), S0 = Tap water, S1 = 42.8 mM, S2 = 85.6 mM, S3 = 128.3 mM, Melatonin (M), M0 = 0 µM, M1 = 50 µM, M2 = 100 µM, Bacterial inoculation (B), B0 = non-inoculation, B1 = inoculation



Fig. 2 Pictures of the experiment. Salinity levels (S), S0 = Tap water, S1 = 42.8 mM, S2 = 85.6 mM, S3 = 128.3 mM, Melatonin (M), M0 = 0 µM, M1 = 50 µM, M2 = 100 µM, Bacterial inoculation (B), B0 = non-inoculation, B1 = inoculation

solution was allowed to incubate at room temperature for 30 min before measuring the absorbance at 415 nm using a spectrophotometer. The calibration curve was constructed using quercetin as the standard.

2.2.3 Physiological measurements

After two weeks of the last treatment of Melatonin foliar application, the fourth fresh fully expanded leaf was collected to determine electrolyte leakage and relative water content.

Determination of electrolyte leakage (EL) Electrolyte leakage was assessed following the method outlined by Jambunathan (2010). For one day, leaf discs with a diameter of 1.6 cm were incubated in 20 ml of deionized water. The initial electrical conductivity (EC_1) of the solution was measured using an EC meter. Subsequently, the plant samples were boiled for 20 min, and the final electrical conductivity (EC_2) was recorded. The percentage of leakage was calculated using the formula: (EC_1/ $EC_2 \times 100$).

Determination of relative water content (RWC) Relative water content (RWC) of leaf tissue was determined according to the methodology established by Smart and Bingham (1974). To determine the fresh weight (FW), leaf discs from completely expanded Calendula leaves were weighed. They were then immediately dipped in distilled water for the entire night to become turgid, and the turgid weight (TW) was recorded. The discs were then dried for 24 h at 70°C in an oven to determine their dry weight (DW). The following formula was used to determine RWC:

$$\text{RWC } \% = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

2.2.4 Determination of proline

The acid ninhydrin technique was used to estimate the proline content (Bates et al 1973) as follows: Reagents: Acid-ninhydrin was prepared by warming (1.25 g) ninhydrin in (30 ml) glacial acid + (20 ml) 6 M phosphoric acid, with agitation until dissolved (stored at 4° C). Procedure: (0.5g) of leaves were homogenized in 10 ml of 3% aqueous sulfosalicylic acid, then filtered using filter paper. Next, 2 ml of the filtrate was combined with 2 ml of acid ninhydrin + 2 ml of glacial acetic acid in a test tube, and the mixture was heated for 1 h at 100°C. To stop the reaction, the test tube was placed in an ice bath. Subsequently, the reaction mixture was extracted with 4 ml of toluene, 15 to 20 s of vigorous mixing using a test tube stirrer, and the absorbance was read at 520 nm.

2.2.5 Mineral analysis of vegetative growth

Samples of dried herbs that were collected at the end of the experiment and dried in a forced air oven at 70°C were subjected to mineral analysis. The acid-digested solution was used to determine N, P, K, and Na as explained by Thomas et al (1967), 0.2 g of the dry materials were extracted and digested using a sulfuric acid and hydrogen peroxide mixture.

According to Okalebo et al (2002), the colorimeter method (ammonium molybdate) utilizing a spectrophotometer was used to determine the phosphorous concentration, and the micro-Kjeldahl method was used to estimate the total nitrogen content. The potassium concentration was measured using an atomic absorption spectrophotometer (Chapman and Pratt 1982). Sodium was measured using an atomic absorption spectrophotometer as described by Munns et al (2010). Chloride was measured in dry digestion extract by titration with silver nitrate, utilizing Mohr's method, as described by Belcher et al (1957).

2.2.6 Enzyme activity

The extraction of enzymes and soluble proteins was performed by homogenizing 0.5 g of fresh leaves in 4 mL of 0.1 M sodium phosphate buffer (p^H 7.0), supplemented with 1% polyvinylpyrrolidone (PVP) and 0.1 mM EDTA. The homogenate was then centrifuged at 10,000 × g for 20 min at 4°C, and the resulting supernatant was utilized for subsequent analyses. The concentration of soluble proteins was determined using the Bradford method (Bradford 1976).

Polyphenol oxidase (PPO) The polyphenol oxidase (PPO) activity was measured using the procedure outlined by Oktay et al (1995). 100 µl of crude enzyme extract, 600 µl of catechol, and 2.3 ml of a phosphate buffer (0.1 M, pH 6.5) made up the reaction mixture. Measurements of absorbance were made at 420 nm using a spectrophotometer, both at the initiation of the reaction and after one minute.

Peroxidase (POD) Peroxidase (POD) activity was quantified following the protocol established by Dias and Costa (1983), with minor modifications. The assay mixture comprised 10 mL of a 1% (v/v) guaiacol solution, 10 mL of a 0.3% hydrogen peroxide solution, and 80 mL of a 50 mM phosphate buffer at pH 6.6. A total of 2.9 mL of the assay mixture was mixed with 100 µl of crude enzyme to start the reaction. At a wavelength of 470 nm, absorbance was measured every 30 s for three minutes.

2.2.7 Total chlorophyll estimating

Two weeks after the last treatment of melatonin, leaf samples were taken to determine the leaves' chlorophylls

according to Siebeneichler et al (2019). 0.1 g of chopped leaves was incubated in 5 ml of N, N-dimethylformamide (DMF) for 24 h. The pigment quantification was performed by spectrophotometry, with wavelengths of 665 nm and 646 nm for chlorophylls.

$$\text{Total chlorophyll} = 7.04^{*}A_{665} - 20.27^{*}A_{646}$$

wherein: A = wavelengths.

2.3 Statistical analysis

The study employed a randomized complete block design (RCBD) for its experimental setup. Statistical analyses were conducted using the CoStat Package Program (Version 6.303; CoHort Software, USA). The data underwent evaluation through a three-way analysis of variance (ANOVA-3) approach. Duncan's Multiple Range Test was utilized to assess the differences in means. All statistical conclusions were drawn at a significance level of $P < 0.05$.

3 Results

3.1 Plant growth

Leaf area increased by 79.49% and 63.83% when Calendula was sprayed with 100 µM melatonin with bacterial inoculation in the first and second seasons, respectively compared to the control. The highest salinity reduced leaf area significantly by 74.30% in season one and 68.09% in season two compared to control. However, spraying 50 or 100 µM melatonin alone or in combination with adding bacteria increased leaf area in both seasons compared to non-using them. At 42.8 and 85.6 mM, spraying 100 µM melatonin and adding bacterial mixture increased leaf area by 81.07% and 83.63% respectively in 1st season. And by 57.94% and 82.39% respectively in 2nd season compared to non-using them under the same salinity level (Fig. 3).

The dry weight of the shoot increased significantly by spraying 50 or 100 µM melatonin and adding bacteria, with a non-significant difference with them in the first season under non-saline conditions. In the second season, spraying 50 µM with bacterial inoculation increased it two-fold significantly compared to the control. However irrigated Calendula with 128.3 mM decreased shoot dry weight, but spraying 50 µM with bacterial inoculation increased it about three-fold during the first season and about two-fold in the second season. At 42.8 and 85.6 mM, treatment of (100 µM + bacteria) increased dry weight significantly in both seasons compared to non-using them under the same level (Fig. 3).

3.2 Inflorescences parameters

As shown in Fig. 4 the Calendula inflorescences count increased significantly by using bacteria plus spraying 50 or 100 µM of melatonin with a non-significant difference found between both concentrations under normal irrigation in the two successive seasons also, the count of inflorescences

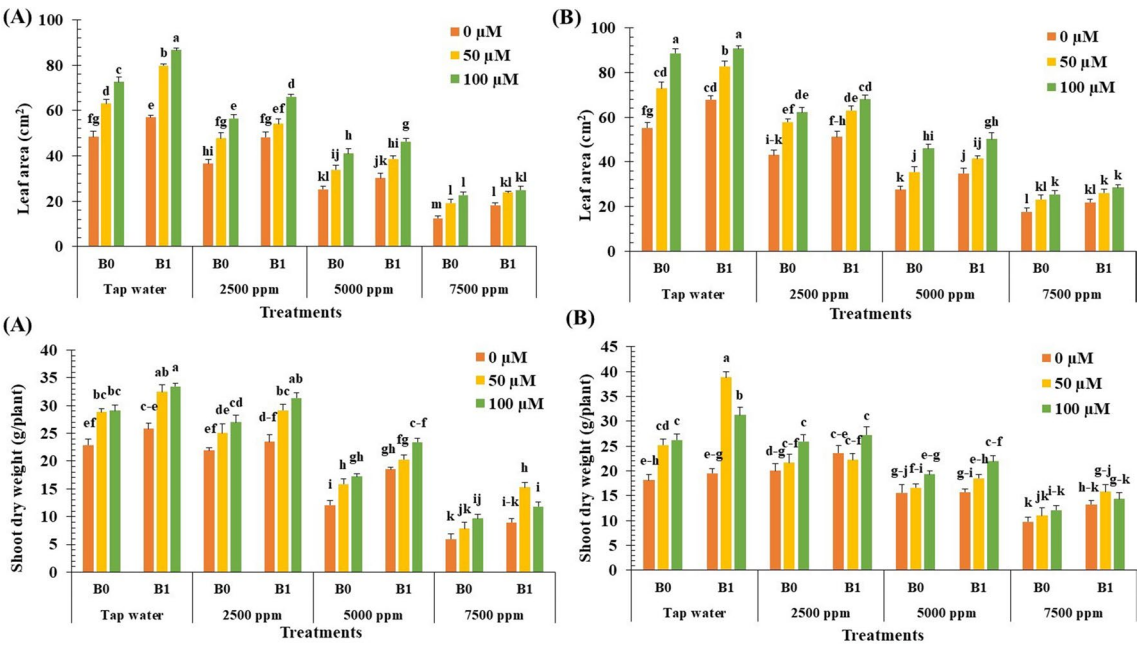


Fig. 3 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on Leaf area and shoot dry weight of *Calendula officinalis* in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

increased more than two-fold compared to the control group. However the highest concentration of saline water (128.3 mM) without any treatments reduced inflorescence count significantly by 60.03% during the first season and 55.11% in the second season. Spraying 100 µM of melatonin with bacterial inoculation increased inflorescence count significantly by two-fold and half in the first season and three-fold in the second season. Under saline concentrations of 42.8 and 85.6 mM, using 100 µM of melatonin with bacterial addition increased the count of inflorescences significantly compared to non-using them or using one of them individually.

There is a significant increase in inflorescence diameter when Calendula treated with 50 µM of melatonin with bacterial inoculation to 9.06 cm and 8.28 cm in the 2022/23 and 2023/24 seasons respectively compared to control under non-saline irrigation. However, there was a non-significant difference between spraying with 50 or 100 µM with bacteria in the second season only. Under 128.3 mM of saline water, the inflorescence diameter decreased significantly to 4.35 cm and 3.59 cm in both seasons respectively, but inoculation of Calendula with bacteria and spraying it with 50 µM melatonin significantly increased inflorescence diameter to 5.7 cm in season one compared to non-using them. However, in the second season, there was a non-significant difference between spraying 50 or 100 µM under the same conditions (Fig. 4).

The dry weight of Calendula inflorescences as shown in Fig. 4 increased significantly when sprayed with 100 µM melatonin and the addition of bacteria to reach 8.17 and 9.97 g/plant from control (4.45 and 6.49 g/plant) in 1st and 2nd seasons respectively under irrigation with tap water. However, a non-significant difference was found between spraying 50 or 100 µM under the same conditions in the second season. Under 128.3 mM of saline water, there was a non-significant difference between using 50 or 100 µM melatonin with bacterial inoculation which increased inflorescences' dry weight significantly as 2.38 and 2.64 g/plant in 1st season respectively compared to non-using

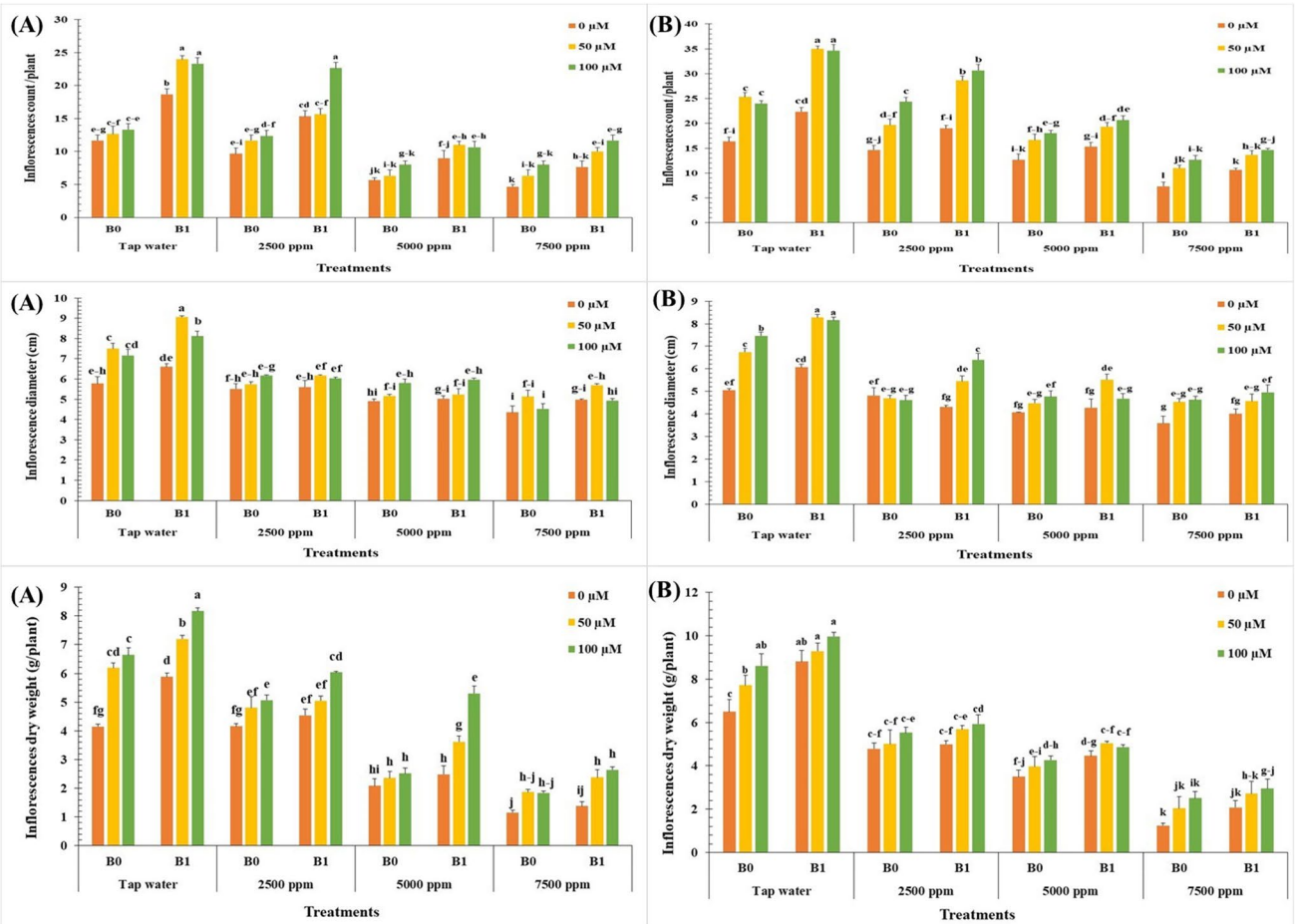


Fig. 4 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on inflorescences count, diameter, and dry weight of *Calendula officinalis* in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

them (1.16 g/plant). However, in the second season, using 100 µM melatonin with bacterial inoculation increased the inflorescence's dry weight significantly to 2.95 g per plant compared to non-using them (1.24 g/plant). In the first season, spraying 100 µM melatonin with bacterial inoculation significantly increased inflorescences dry weight under 42.8 and 85.6 mM (6.03 and 5.30 g/plant) compared to non-using them (4.14 and 2.09 g/plant). Also, it was increased in the second season (5.93 and 4.87 g/plant).

The highest flavonoid content in Calendula petals as shown in Fig. 5 was obtained by using 50 µM of melatonin with bacterial addition under the irrigation with 128.3 mM, which increased significantly by 34.87% compared to irrigation with the same concentration without any treatments in the first season. However, using 100 µM of melatonin with bacterial addition under 128.3 mM increased total flavonoids by 29.39% compared to irrigation with the same concentration without any treatments in the second season with a non-significant difference between melatonin concentrations (50 or 100 µM). Non-saline irrigation without any treatments gave the lowest content of flavonoids which decreased by 62.35% and 72.85% in season one and season two respectively compared to the highest treatment.

A significant increase of 42.71% in total carotenoids in petals in the 1st season and 60.19% in the 2nd season when plants were sprayed with 100 µM of melatonin with bacterial mixture addition compared to those left without any treatments, in case of applying the regular irrigation. This treatment also showed its superiority in increasing the level of carotenoids under saline irrigation levels, where it increased significantly by 35.86%, 27.63%, and 33.87% under saline irrigation with 42.8, 85.6, and 128.3 mM, respectively in the first season, and 30.88%, 30.92% and 25.28% in the second season compared to the treatment left without any treatments under the same irrigation level. It is also noted that there was a non-significant difference between spraying treatments of 50 µM and 100 µM of melatonin with the addition of bacteria when using irrigation with 85.6 and 128.3 mM (Fig. 5).

3.3 Chlorophyll content

Data in Fig. 6 showed that 100 µM melatonin with bacterial inoculation increased total chlorophyll in Calendula leaves significantly under non-saline irrigation by 78.09% in 1st season and 67.64% in 2nd season compared with control. The highest salinity (128.3 mM) without any treatments decreased total chlorophyll by 80.95% and 77.94% in two successive seasons respectively, While the foliar spraying with 100 µM melatonin and adding the bacterial mixture, increased chlorophyll by 70% and 53.33% in two successive seasons respectively compared to non-using them. Under 85.6 mM, there is a non-significant difference between spraying 50 or 100 µM melatonin only or with bacterial

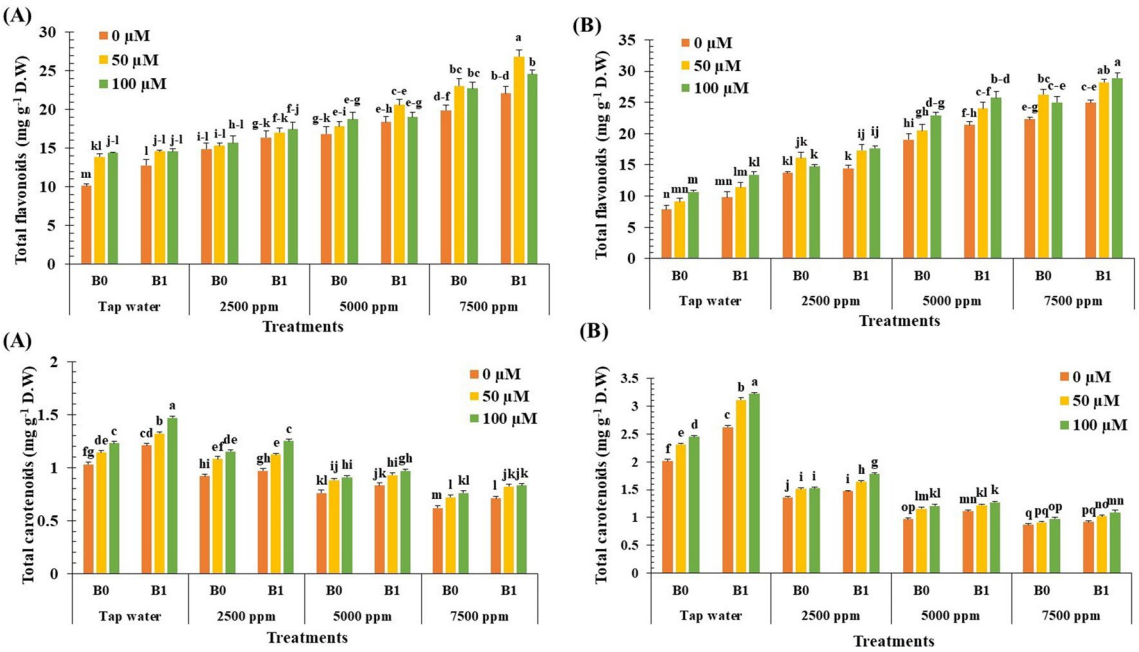


Fig. 5 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on total flavonoids and total carotenoids of *Calendula officinalis* petals in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

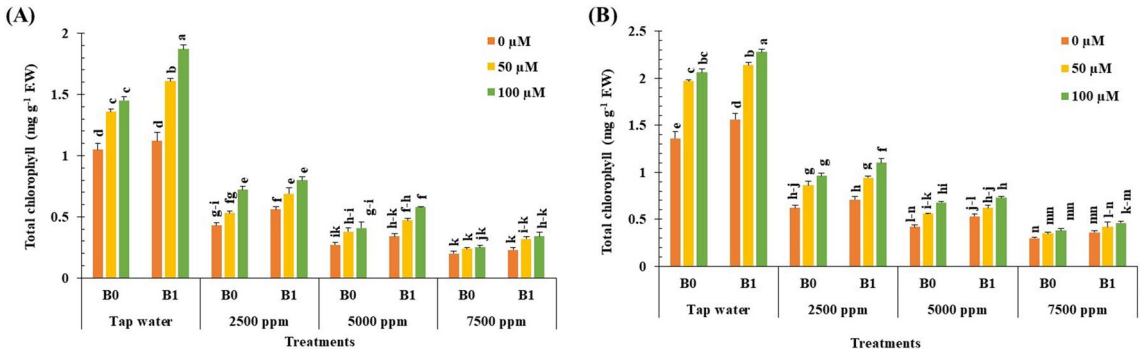


Fig. 6 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on total chlorophyll of *Calendula officinalis* leaves in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

addition in two successive seasons. As for 42.8 mM, 100 µM melatonin with bacterial inoculation increased total chlorophyll by 86.04% with a non-significant difference with a concentration of 50 µM in the first season and by 77.41% in the second season compared to non-using them.

3.4 Mineral analysis and proline content

Adding bacterial mixture with spraying 100 µM melatonin increased nitrogen in Calendula by 22.41% and 37.63% in the first and the second seasons respectively compared to non-using them at 42.8 mM. Also, under 85.6 mM using a bacterial mixture with spraying 100 µM melatonin increased it by 35.50% in the first season and 68.26% in the second season compared to saline irrigation only. Under 128.3 mM, using 50 or 100µM + bacteria increased nitrogen significantly compared to non-using them in both seasons. In the first season only, there was a non-significant difference between spraying with 50 or 100µM + bacteria in all irrigation levels (Fig. 7).

Figure 7 showed a significant increase in phosphorus when Calendula sprayed with 100 µM melatonin with bacterial addition at normal irrigation which increased by 41.37% and 66.66% in comparison to the control in the first and second seasons, respectively. The highest saline water (128.3 mM) decreased phosphorus significantly by 62.06% and 51.85% in 1st and 2nd seasons respectively. However, sprayed 50 µM melatonin with bacterial addition increased phosphorus two-fold compared to non-using them in the first season, and sprayed 100 µM melatonin with bacterial addition increased phosphorus by 61.53% compared to non-using them in the second season. At 42.8 and 85.6 mM, 100 µM melatonin with bacterial inoculation increased phosphorus significantly compared to irrigated without them in both seasons which increased by 37.50% and 58.82% in season one, and 40% and 55.55% in season two, respectively. There was a non-significant difference between spraying with 50 or 100 µM melatonin with bacterial inoculation at all salinity levels.

Figure 8 showed that spraying 100 µM melatonin and adding bacteria gave the highest potassium percentage which increased significantly by 23.86% and 40.54% in the first and second seasons respectively under non-saline irrigation. However, there was a non-significant difference between using 50 or 100 µM melatonin with bacteria in the first season only. Although potassium decreased under saline irrigation, foliar spraying with 100 µM melatonin with bacterial inoculation increased it significantly by 60.50% and 29.41% at 42.8 mM. Also, at 85.6 mM increased significantly by 64.58% and 35.23% in the first and second seasons respectively compared to non-using them under the same level of salinity. The lowest percentage of potassium was obtained by using 128.3 mM without any treatments. However, using bacteria by spraying 100 µM melatonin increased it significantly by 124% in 1st season and 47.61% in 2nd season compared to irrigation only.

Although the highest saline water (128.3 mM) increased sodium percentage significantly, the treatment of adding bacteria with 100 µM melatonin decreased it by 24.52 and 26.01% in the first and second seasons respectively. Also, this treatment decreased it by 40% and 36.50% at 42.8 mM, as well as decreased it by 25.95% and 26.08% at 85.6 mM in the first and second seasons respectively. There is a non-significant difference between 50 or 100 µM melatonin with adding bacteria at all irrigation levels, which has the same effect of decreasing sodium concentration. The lowest sodium percentage was at non-saline irrigation with a non-significant difference between treatments (Fig. 8).

Chloride percentage increased significantly about nine-folds and six-folds in 1st season and 2nd season respectively when calendula irrigated with 128.3 mM without any treatments compared to the control. However, spraying 100 µM of melatonin and adding bacterial inoculation decreased it by 21% in 1st season and 20.38% in 2nd season. Also, at 42.8 and 85.6 mM spraying 100 µM of melatonin and adding bacteria decreased Cl% by 23.97 and 24% in the first season

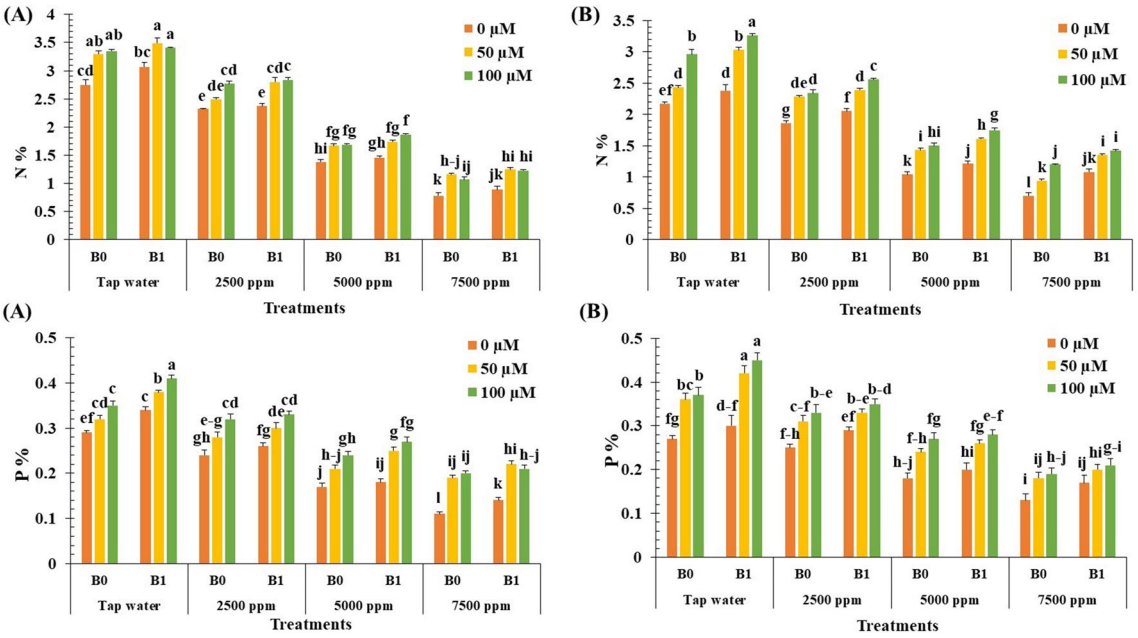


Fig. 7 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on a percentage of nitrogen and phosphorus of *Calendula officinalis* leaves in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

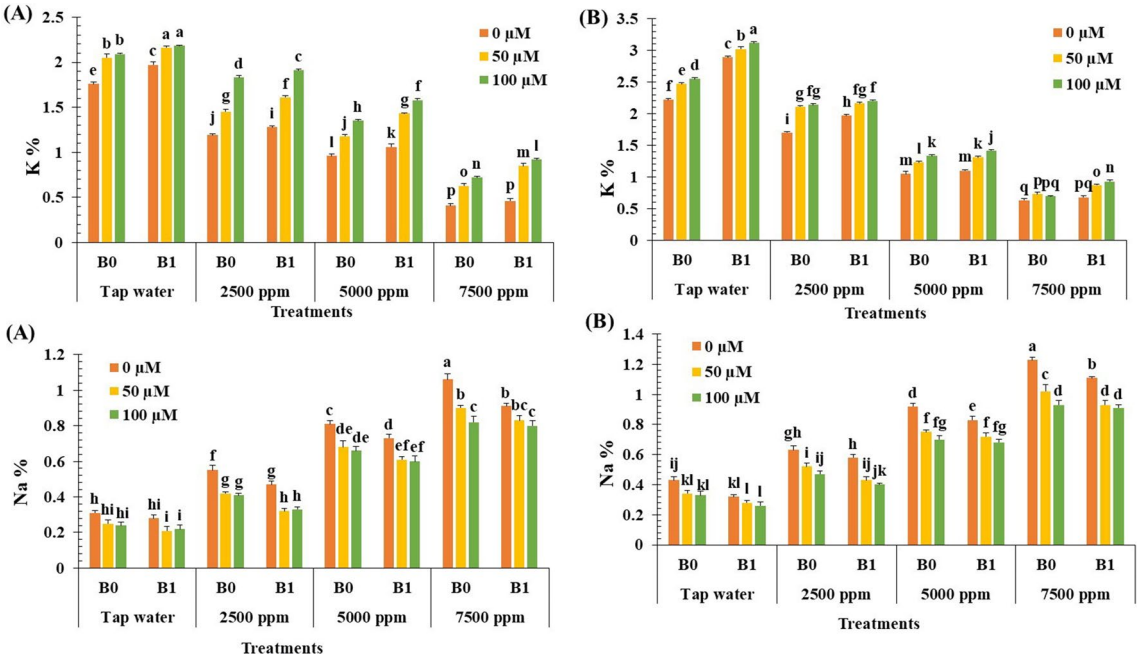


Fig. 8 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on a percentage of potassium and sodium of *Calendula officinalis* leaves in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

respectively. And by 30.24 and 24.19% in the second season respectively compared to non-using them under the same level of salinity. The lowest chloride percentage was at non-saline irrigation with a non-significant difference between treatments (Fig. 9).

Foliar application of 100 µM melatonin with bacterial inoculation at 128.3 mM increased proline significantly by 32.19% in the first season but in the second season, 50 µM melatonin with bacterial inoculation increased proline significantly by 36.16% compared to non-using them. Under saline irrigation with 85.6 mM, 100 µM melatonin with adding bacteria increased proline significantly by 26.43% and 23.89% in the first and second seasons respectively compared with irrigation only. In the first season, adding bacterial mixture alone or adding it with melatonin increased proline at 42.8 mM. However, in the second season spraying 100 µM melatonin with bacterial addition increased it significantly compared to other treatments under the same level of salinity. There is a non-significant difference in proline content under normal irrigation which records the lowest concentrations (Fig. 9).

3.5 Enzymes activity

Figure 10. showed that peroxidase (POD) activity increased significantly by spraying 100 µM of melatonin with bacterial inoculation under non-saline irrigation to 6.71 and 3.49 Units.mg^{-1} protein min^{-1} in the first and second seasons respectively compared to control. The lowest activity of peroxidase was obtained by using saline water at 128.3 mM without any treatment which decreased significantly by 71.47 and 63.88% in two successive seasons compared to control. However, adding bacteria by spraying melatonin at 100 µM increased POD activity by 21.17 and 25.64% in two successive seasons compared to non-using them. Under 85.6 mM, using melatonin at 100 µM with bacteria increased peroxidase activity by 49.56 and 46.60% in two successive seasons compared to non-using them. In the first season under 42.8 mM, spraying 50 or 100 µM only increased POD activity. But in the second season, there is a non-significant difference between spraying 50 or 100 µM with bacteria and spraying 100 µM only.

On the other hand, polyphenol oxidase (PPO) activity was reduced significantly when Calendula was irrigated with regular water without treatments, while it increased to 30.82% by using bacteria with 100 µM melatonin in the first season and 55.17% by using bacteria with 50 µM melatonin in the second season. The highest activity of PPO was under 42.8 mM concentration when plants sprayed with 50 µM melatonin with bacterial addition in 1st season by 85.81%, and 100 µM melatonin with bacterial addition in 2nd season by 40.02% compared with non-using them. Under irrigated with saline water by 85.6 and 128.3 mM, foliar spraying with 100 µM melatonin with bacterial inoculation increased polyphenol oxidase activity by 84.55% and 18.25% in the first season respectively, whereas 75.97% and 46.60% in the

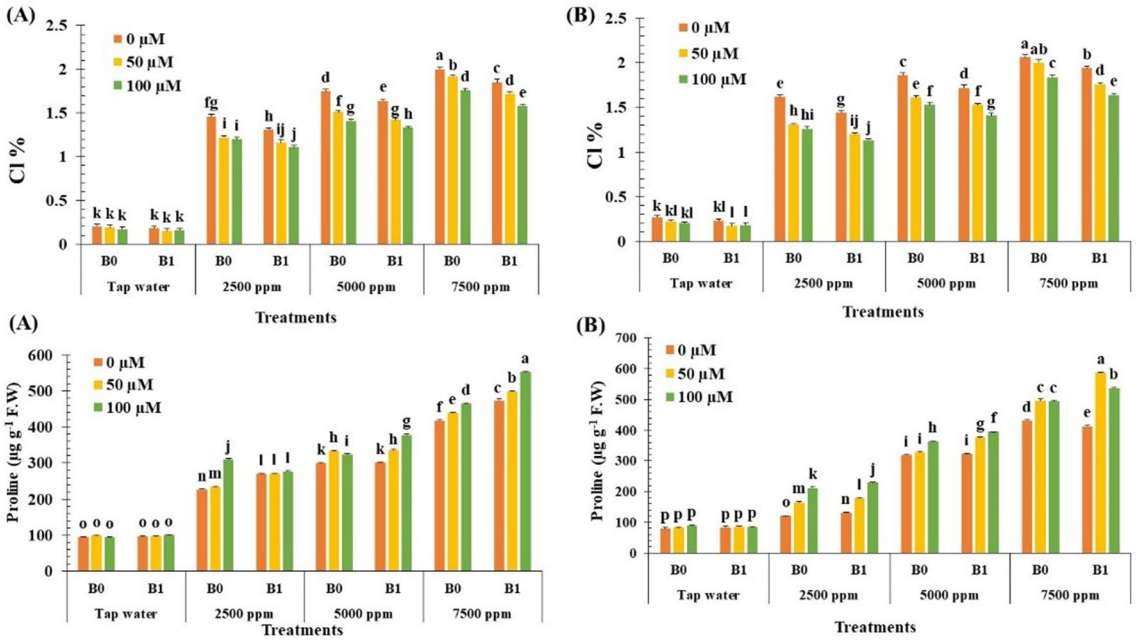


Fig. 9 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on a percentage of chloride and proline content of *Calendula officinalis* leaves in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

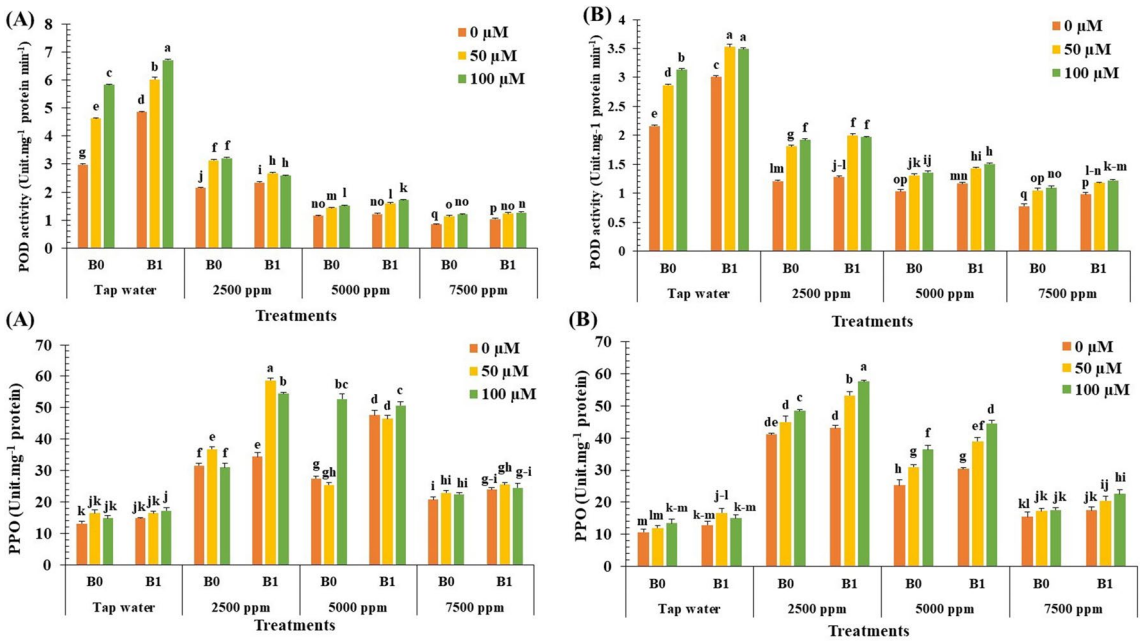


Fig. 10 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on peroxidase (POD) and polyphenol oxidase (PPO) activity of *Calendula officinalis* leaves in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

second season respectively compared to non-using them under the same level of salinity.

3.6 Physiological parameters

Figure 11 showed that foliar spraying of melatonin only or with bacterial inoculation increased the percentage of the relative water content of Calendula leaves under normal irrigation. The highest percentage was obtained by using bacteria with 100 µM melatonin (98.66 and 98.88%) compared to control (86.01 and 88.14%) in two successive seasons respectively, with a non-significant difference between it and other concentrations whether used alone or with bacteria. Irrigation with the highest concentration (128.3 mM) only gave the lowest percentage of relative water content which decreased significantly to 36.08% in 1st season and 44.57% in 2nd season. Under salinity with 42.8 mM, the addition of bacteria by spraying 50 or 100 µM melatonin increased relative water content significantly compared to irrigation alone. In the first season under 85.6 mM, using 100 µM of melatonin with bacterial addition increased RWC% significantly to 77.81% compared to irrigation only (52.27%) but, in the second season there is a non-significant difference between spraying melatonin only, or inoculation of bacteria only, or both of them together.

Electrolyte leakage significantly increased when Calendula was irrigated with 128.3 mM without any treatments which reached 90.53% and 88.84% in the first and second seasons respectively, but using 50 µM of melatonin with bacterial addition decreased it to 79.47% and 74.16% in the first and second season respectively compared to non-using them. Under 85.6 mM, there is a non-significant difference between spraying 100 µM melatonin alone or with bacteria which decreased EL% in both seasons compared to irrigation alone. On the other hand, there is a non-significant difference between all treatments under normal irrigation in both seasons (Fig. 11).

4 Discussion

Salt stress reduces plant growth by impairing water uptake, leading to slower growth. Additionally, salt can enter the transpiration stream, potentially causing cellular damage in the transpiring leaves (Parihar et al 2015). Many previous studies have indicated the importance of melatonin or using bio-stimulants like *Pseudomonas fluorescens*, *Bacillus megaterium*, and *Azotobacter chroococcum* in enhancing plant development and providing protection against salinity in numerous crops (Abdelaziz 2022; Fu et al 2010; Wang et al 2018) but, there is insufficient information about the interaction between them under salinity. The results showed that the combination of melatonin and bacterial mixture significantly improved shoot dry weight and leaf area in non-saline and saline irrigation to 85.6 mM through the positive effect of interaction in

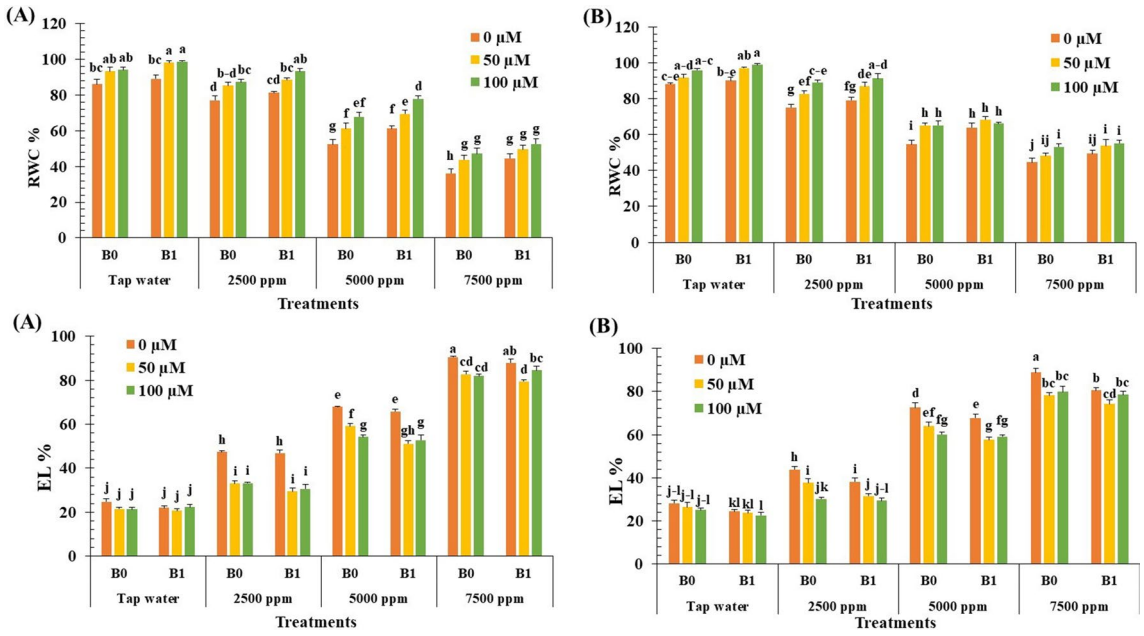


Fig. 11 Effect of the interaction between salinity levels (ppm), foliar spraying of melatonin (µM), and bio-stimulants (inoculation (B1) or non-inoculation (B0)) on relative water content (RWC) and electrolyte leakage (EL) of *Calendula officinalis* in the first season **A** and second season **B**. Bars with identical lowercase letters are not significant at $P < 0.05$ level according to Duncan's multiple range test. Data are mean value ± SE

the regulation of ion homeostasis and the enhancement of chlorophyll concentration and photosynthetic activity.

Salinity stress -as shown in this study- adversely affects the quality of Calendula inflorescences by reducing their number, size, and dry weight, similar to findings in *Calendula officinalis* (Khalid and da Silva 2010). This decline is attributed to the plant's inability to adjust and mitigate toxic effects osmotically (Abdel-Mola and Ahmed 2020). However, bacterial inoculation combined with melatonin spraying improves inflorescence quality under normal and saline conditions. It is expected that exogenously applied melatonin boosts endogenous levels of it, indole-3-acetic acid, gibberellins, and cytokinins, essential for prolonging floral retention and enhancing flower yield (Yousif et al 2024). Also, PGPR influences the hormonal balance within plants which can lead to enhanced flowering under stress (Nadeem et al 2015).

Excessive cytoplasmic NaCl disrupts ion balance, inhibiting plant growth and development (Yuan et al 2016). In this study, leaf Na^+ and Cl^- concentrations increased significantly under saline conditions, similar to findings in Calendula leaves (Abdel-Mola and Ahmed 2020). This may result from plants redistributing salt ions into vacuoles or different tissues as a salinity stress adaptation (Zhu 2003). Yu et al (2018) reported that exogenous melatonin promotes the breakdown of triacylglycerols, β-oxidation of fatty acids, and energy turnover during salinity stress, thereby supporting plasma membrane H^+-ATPase activity and K^+/Na^+ homeostasis. The reduction in sodium and chloride content associated with varying levels of melatonin may be linked to increased Ca^{2+-}ATPase activity at the plasma membrane, which provides the necessary energy for the removal of excess sodium (Liu et al 2020). These findings are supported by (Eisa et al 2023), who noted that Mel treatment mitigated ion toxicity in Ranunculus under salt stress. Additionally, *Bacillus megaterium* has been shown to decrease Na^+ levels while increasing Ca^{2+} concentrations (Fan et al 2016). This study shows that applying melatonin with bacterial inoculation significantly increased N, P, and K availability and mitigated saline stress. Melatonin enhances the expression of ion-channel genes that regulate nutrient transport across cell membranes. This regulation improves the efficiency of nutrient uptake processes, allowing plants to absorb more N, P, and K in saline conditions (Farag et al 2020). Moreover, microbial bio-stimulants enhance phosphorus and potassium solubilization and nitrogen availability by producing plant growth-promoting metabolites such as phytohormones, siderophores, ACC deaminase, and anti-phytopathogenic compounds under salinity (Bidalia et al 2019).

The study's findings showed a considerable decrease in RWC and a significant increase in electrolyte leakage caused by elevated salt stress levels in Calendula leaves. This decline in RWC likely contributed to decreased growth factors across various parameters. These findings

are consistent with previous research conducted on *Ranunculus asiaticus* (Eisa et al 2023). The observed increase in RWC in Calendula may result from melatonin's ability to modulate stomatal behavior, reducing water loss through regulated stomatal opening and closing (Khan et al 2024). Improved water absorption is linked to melatonin's role in enhancing cell membrane protection and increasing cuticle thickness, which minimizes water loss. Additionally, melatonin preserves a healthy water balance and maintains turgor pressure under stress conditions, enhancing stress tolerance (Liu et al 2015; Castañares and Bouzo 2019). Reduced electrolyte leakage, a marker of membrane integrity, indicates melatonin's role especially when combined with bacteria in retaining potassium (K +) during stress (Parkash and Singh 2020). Additionally, plant growth-promoting rhizobacteria (PGPR) improve water status under salinity by enhancing RWC, reducing oxidative damage, and minimizing electrolyte leakage (EL) in leaves (Prittesh et al 2020; Zarei 2022).

This study observed a significant decline in total chlorophyll content, particularly under high salinity. Elevated Na^+ levels are linked to hindered synthesis or accelerated degradation of photosynthetic pigments and negatively impact plant growth (Muller et al 2014). However, the significant enhancement of total chlorophyll by using melatonin and bacterial mixture can be attributed to two synergistic effects: the stimulation of chlorophyll biosynthesis by exogenous melatonin and the mitigation of chlorophyll degradation, likely due to its antioxidative and stress-alleviating properties (Liang et al 2019). Moreover, *Bacillus megaterium* inoculation in *Phaseolus vulgaris* has been reported to mitigate salinity effects and enhance photosynthetic pigments under salt stress (Abdelmoteleb and Gonzalez-Mendoza 2020).

Plants use osmotic regulators as adaptive strategies to mitigate stress and maintain intracellular stability, protecting cells from salinity's adverse effects (Zhu 2001). Proline stabilizes membranes, proteins and regulates gene expression under saline conditions to maintain redox balance (Ferchichi et al 2018). Studies showed that melatonin-induced osmolyte accumulation lowers cellular osmotic potential, enhancing osmotic adjustment and water content under stress (Castañares and Bouzo 2019). This study corroborates these findings by demonstrating that melatonin treatment enhances proline accumulation under saline conditions, consistent with Eisa et al (2023). Additionally, *Pseudomonas fluorescens* increases proline levels in wheat under saline soils (Kadmiri et al 2018), while *Bacillus* sp. enhances proline in maize (Ullah and Bano 2015).

In this study, Mel with bacterial bio-stimulants significantly increased POD activity under stress conditions. Exogenous application of melatonin reduces ROS formation by enhancing antioxidant activity and directly scavenging ROS (Shi et al 2015). Similarly, Eisa et al (2023) found that Mel improves salt tolerance by upregulating antioxidant enzyme gene expression and preventing biological macromolecule degradation, leading to increased enzyme activity under high salinity. In this investigation, polyphenol oxidase (PPO) activity increased under low to moderate salinity levels, likely as a defensive response to mitigate the harmful effects of ROS. PPO plays a role in ROS detoxification, reducing oxidative damage to plant cells (Hasanuzzaman et al 2021). Moreover, elevated PPO activity may reflect the plant's strategy to strengthen cell walls by producing phenolic compounds that cross-link cell wall components, enhancing structural support and rigidity. This reinforcement helps plants resist osmotic stress caused by salinity (Boeckx et al 2015). Previous studies also highlight increased peroxidase activity, antioxidative enzymes, and osmolytes in plants inoculated with *Pseudomonas fluorescens* (Kadmiri et al 2018), *Bacillus* sp. (Ullah and Bano 2015), and *Azotobacter chroococcum* under saline conditions (Abdel Latef et al 2020).

Elevated NaCl levels as shown in this study significantly reduce carotenoid content, likely due to the degradation of carotenoids under salt stress or suppressed expression of carotenoid biosynthetic pathway genes. This was observed in Calendula (Azizi et al 2021). Maintaining high carotenoid levels under salinity is vital for preserving the quality of marigold flowers. Melatonin application with bacterial inoculation effectively reduces salinity-induced carotenoid degradation. Similarly, exogenous melatonin has been shown to enhance carotenoid levels in Ranunculus (Eisa et al 2023) and Fenugreek (Esboei et al. 2022). Additionally, this study found that flavonoid concentrations increased with saline irrigation, consistent with findings by Guzman and Marques (2023). Melatonin treatment significantly increased flavonoid levels, supporting findings by Esboei et al (2022). Elevated flavonoid concentrations play a pivotal role in maintaining Na^+/K^+ ion balance and mitigating stress effects by regulating hormonal and transcriptional processes, particularly those linked to abscisic acid signaling (Jan et al 2021). Moreover, bacterial genera such as Bacillus and Pseudomonas have been reported to boost flavonoid accumulation under salinity stress (Qin et al 2014). These results underscore the critical role of carotenoids and flavonoids in plant adaptation to salinity stress, emphasizing that melatonin and beneficial microbial inoculants are promising tools for enhancing flower quality and marigold resilience.

5 Conclusion

This study demonstrates that saline irrigation negatively impacts the growth, flowering, and physiological processes of *Calendula officinalis*. However, the application

of melatonin, particularly at a concentration of 100 µM, and bacterial inoculation with *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens*, can effectively mitigate these adverse effects. Melatonin and bacterial inoculation enhance vegetative growth and inflorescence production, even under saline conditions. These treatments improve nutrient uptake, water content, and enzymatic activity, leading to enhanced overall plant health and productivity. The combined application of melatonin at 100 µM and bacterial inoculation consistently yielded the most promising results, suggesting a synergistic effect in alleviating salt stress and promoting the growth and flowering of the Calendula plant. These findings underscore the potential of melatonin and bacterial bio-stimulants as valuable tools for sustainable Calendula cultivation in saline environments. Future research could conduct detailed mechanistic studies to understand how melatonin and bacterial bio-stimulants interact at the molecular level. This could involve examining gene expression changes related to salt stress tolerance, nutrient uptake, and enzymatic activity.

Acknowledgements The authors would like to extend their sincere appreciation to the Department of Horticulture, Faculty of Agriculture, Ain Shams University.

Author contributions All authors contributed to this study's conception and design. Omniea Mohsen Allam performed material preparation, data collection, and analysis. Omniea Mohsen Allam wrote the first draft of the manuscript, and all authors commented on previous versions. All authors read and approved the final manuscript.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval No ethical approval was needed for this study.

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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