

Article

Effects of Water Stress and Modern Biostimulants on Growth and Quality Characteristics of Mint

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Abstract: Natural biostimulants combine different elicitors that may influence economic properties of herbal crops, such as mint. Mint (*Mentha longifolia* L.) plants were subjected to three water levels based on container substrate capacity (CSC; 100% CSC, 70% CSC, and 50% CSC) and/or applications of four biostimulants (CRADLE™, Mobilizer™, Nanozim De'Lite™ [ND], and Nanozim NXT™ [NN]). ND and NN exhibited higher vegetative growth and root dry weight than the control (without biostimulants) and other treatments. NN produced the highest fresh and dry mint yields under all water levels. Irrigation water-use efficiency (IWUE) of NN was highest (2.78 kg m⁻³) with 70% CSC, whereas the control produced the lowest IWUE (1.85 kg m⁻³) with 100% CSC. Biostimulants boosted physiological and metabolic responses, including gas exchange, leaf water potential, relative water content, and proline accumulation of stressed plants. NN treatment with 70% CSC had the highest essential oil (EO) ratio (3.35%). Under 70% and 50% CSC with NN treatment, the proportion of 1,8-cineol increased and that of pulegone decreased in EOs. Increased antioxidant activities, reduced H₂O₂ levels, and increased catalase and superoxide dismutase activities were observed. Applications of ND and NN during water stress conditions increased economic and medicinal properties of mint EOs with applications in the agricultural and pharmaceutical industries.

Keywords: *Mentha longifolia*; biostimulants; *Ascophyllum nodosum*; humic acid; antioxidants

1. Introduction

Mint plants have a long history as traditional medicinal plants [1]. *Mentha longifolia* L. belongs to the family Lamiaceae and naturally occurs in Egypt, Saudi Arabia, and most Arabian countries. The fresh/dried plants are mainly used as an herbal medicine for the treatment of indigestion, menstrual pain, coughs, asthma, fever, and headaches [2,3]. The fresh leaves are used in soft drinks and as garnishes for salads in some countries. The essential oil (EO) is used in the pharmaceutical, cosmetic, and food industries [4]. The EOs exhibit strong antimicrobial activity against several microorganisms [3].

Water stress is one of the major limiting factors for agriculture and food safety worldwide [5]. This stress causes reduced vegetative growth and great losses to farmers. Different studies have focused

on the effects of water stress on the growth parameters and EO yield. Zade et al. [6] reported that water stress decreased peppermint plant fresh and dry weight, leaf number, plant height, and root dry weight but nonetheless increased EOs compared to that of normal irrigation in greenhouse and field experiments. Figueroa-Pérez et al. [7] showed that water stress decreased fresh and dry weights of peppermint but increased composition of plant secondary metabolites and antioxidant capacity. Ekren et al. [8] reported that plant height and yield of purple basil were negatively affected by water stress, whereas the EO content increased and irrigation water-use efficiencies were not significantly affected. Shormin et al. [9] showed that the harmful effects of water stress on Japanese mint yield could not be compensated by high nitrogen quantities. Farahani et al. [10] also reported the highest content of EO in balm occurred at 60% field capacity (FC). However, in other studies, Khorasaninejad et al. [11] showed that water stress had negative effects on some growth parameters and EO content of peppermint plants. Razmjoo et al. [12] found that this stress reduced some growth parameters and EO content of chamomile.

Several approaches have been applied to control water stress, such as the use of biostimulants. Modern biostimulants have been produced to increase the productivity and the quality of horticultural crops and help the plants tolerate stress conditions. Some of these biostimulants are mixtures of seaweed extracts, humic acid, and macro and micro elements, whereas other products contain mixtures of mycorrhiza and seaweed extracts, as well as other micro elements. Seaweed extracts work as elicitors for plant secondary metabolites, including EOs and may increase the pharmaceutical properties against microorganisms [13,14]. However, the effects of the mixtures of seaweed extracts and other elicitors, such as humic acid and specific minerals have not been investigated for mint plants. Further, water stress may cause significant changes in the EO composition and these changes might cause parallel changes in the antimicrobial properties of respective EOs.

In this investigation, our goal was to determine the effects of water stress and commercial biostimulants on the growth, physiology, secondary metabolites, and antioxidant activities of mint (*Mentha longifolia* L.). We propose that these natural biostimulants modulate growth, EO ratio, and EO constituents, leading to enhanced bioactivity of mint plants. These effects indeed have the potential to have future agricultural industry applications.

2. Materials and Methods

2.1. Plant Material

Uniformly rooted cuttings of mint (*Mentha longifolia* L.) were brought from nurseries of the Alexandria University farm in February 2018 and 2019 (as two successive growing seasons). The species was identified and vouchered by Hosam Elansary in the Faculty of Agriculture, Alexandria University. The sandy soil (75.5%, 13.2%, and 11.3% of sand, silt and clay, respectively) samples were air dried and sieved with a 2 mm mesh. The soil had an FC of 20.5%, wilting point of 9.6%, electrical conductivity of 0.36 mS cm⁻¹, organic matter of 1.4%, pH of 6.2, total nitrogen of 0.085%, total phosphorus of 0.05%, and total sulfur of 0.03%. After proper soil preparation, the plants were grown in 2.1 L plastic pots containing the natural sandy soil supplemented with Crystalon[®] (65 kg N ha⁻¹ as urea, 40 kg P₂O₅ ha⁻¹ as triple superphosphate, 34 kg K₂O ha⁻¹ as potassium sulfate, and 2 g L⁻¹ media) in the greenhouse. The temperature inside the greenhouse ranged between 15.0 °C (night) and 27.3 °C (day) and the relative humidity ranged between 67% and 72% during the growing period. The photosynthetic active radiation was approximately 1000 μmol m⁻² s⁻¹ at noon. Daily watering by drip irrigation was applied to reach the full pot substrate FC. Pots were irrigated equally for 30 days after transplantation (DAT). Mint plants were harvested at 90 DAT. Container substrate capacity (CSC) is the maximum amount of water that can be retained by the substrate after the discharge because of gravity [15]. Before planting, the gravimetric method was used to determine CSC or FC by watering the plants to saturate the soil then the pots were left to drain for 60 min and the volume of drained water was quantified and the difference between the supplied and drained water volumes were considered the volumetric water

retained by the soil (i.e., CSC). The amount of water applied (AWA) to compensate for the soil water deficit to reach the FC is calculated as follows:

$$AWA = (CSC - \theta_v) D A \quad (1)$$

where θ_v is soil water content at the irrigation event, D is the soil depth, and A is the surface area of the pot.

2.2. Treatments

The plants were subjected to three watering levels of CSC (100%, 70%, and 50%) after 30 DAT and/or single biostimulant of four commercial biostimulants, namely, CRADLE™, Mobilizer™, Nanozim NXT™, and Nanozim De'Lite™ (Biostadt, Mumbai, India). CRADLE (CR) powder is a mycorrhizal biofertilizer developed by InGene Organics, India and was used at $g L^{-1}$ growing soil. Mobilizer (Mob) is a granular mycorrhizal biofertilizer mixed with kelp seaweed extract (*Macrocystis pyrifera*), humic acid, and amino acids and was applied at $g L^{-1}$ growing soil. Nanozim De'Lite (ND) is a granular formulation of 25% ($w w^{-1}$) seaweed (*Ascophyllum nodosum*), 25% (w/w) carbohydrates, 2% ($w w^{-1}$) amino acid, and 1% (w/w) potassium (K_2O), and was used at $1 g L^{-1}$ with irrigation water. Nanozim NXT (NN) is a liquid mixture of 15% ($w w^{-1}$) seaweed (*Ascophyllum nodosum*), 5% (w/w) humic acid, 1% ($w w^{-1}$) potassium (K_2O), 0.01% ($w w^{-1}$) phosphorus (P_2O_5), 0.05% (w/w) alginic acid, 0.05% ($w w^{-1}$) hydrolyzed protein, and several micronutrients and was applied at $1.5 mL L^{-1}$ of irrigation water. The doses of the biostimulants and method of applications matched the manufacturer recommendations and untreated plants with biostimulants were considered the controls. Plants were grouped into three blocks containing 10 replicates per treatment [3 water levels (100%, 70%, and 50% CSC) \times (4 biostimulants +1 control "without biostimulant") = 15 treatments] and totaling 450 plants (150 plants/block \times 3 blocks) in a completely randomized design.

2.3. Measurements

2.3.1. Morphological and Physiological

Following 9 weeks of treatments, several morphological measurements were determined including leaf number ($plant^{-1}$), leaf area ($cm^2 plant^{-1}$), plant heights (cm), plant fresh weight (g), plant dry weight (g), and root dry weight (g). Irrigation water-use efficiency (IWUE, $kg m^{-3}$) was calculated by dividing the fresh weight of the plant (kg) by the total AWA (m^3) to each treatment during the growing period [16]. A digital area meter was used to determine the leaf area. The dry weights were determined following drying at $35 ^\circ C$ in an oven until reaching a constant weight.

Gas exchange measurements were performed on fully expanded leaves, under clear, sunny conditions using a portable photosynthesis system analyzer (ADC BioScientific, LCi, Bioscientific, Ltd., Hoddesdon, UK) and included photosynthetic rate (A), transpiration rate (E), and stomatal conductance (gs). Leaf midday water potential and midday relative water content were calculated at the end of the experiments at noon following the methods of Elansary et al. [17]. Leaf proline composition was also determined following the methods of Elansary et al. [18].

2.3.2. Essential Oil and Gas Chromatography/Mass Spectrometry (GC/MS)

The EOs were obtained by hydro-distillation of dried leaves for 1 h in Clevenger type glass equipment in the Department of Plant Production, King Saud University. The EO ratio was determined per treatment and the EOs were maintained dry by subjecting samples to anhydrous sodium sulfate, then stored at $4 ^\circ C$. A Thermo Scientific, Trace GC Ultra was used coupled with a mass spectrometer (ISQ). A TG-1MS column (narrow bore, length $30 m \times 0.32 mm$ ID, $0.25 \mu m$ film thickness) was used and the carrier gas was helium. The machine was programmed with a starting temperature of $45 ^\circ C$, then a gradual increase was made to $165 ^\circ C$ ($4 ^\circ C min^{-1}$), followed by an increase to $280 ^\circ C$ ($15 ^\circ C min^{-1}$),

and ending with holding time of 15 min. A 2 μL sample of each EO was injected at 250 °C on a splitless mode flow (1 mL min^{-1}) for splitless time (3 min) followed by another split flow (10 mL min^{-1}). The FID was also accomplished in the same column and program. A homologous series of *n*-alkanes (C_{10} – C_{36}) was used to identify the compounds by the retention time and indices were coupled with a mass spectral search program (NIST Ver. 2.0) and WILEY libraries. Selected references from the literature were also used for comparison purposes [1,13].

2.3.3. Antioxidant Potential

Lipid peroxidation levels expressed as thiobarbituric acid reactive substances (TBARS), catalase (CAT) activity, H_2O_2 , and superoxide dismutase (SOD) activities were quantified in frozen petal tissues [19].

2.4. Statistical Analyses

Data obtained from both years (2018 and 2019) were subjected to analyses of variance (ANOVA) with a completely randomized design to determine the significance of differences among treatments in SPSS Version 22 software. Standard errors (SE) were calculated from the data presenting for the mean of 20 replicates from both years. The least significant differences (LSD) method at $p \leq 0.05$ was used to compare all means at each watering level [20].

3. Results

3.1. Morphological Responses

Plants subjected to different water levels of CSC and/or biostimulants showed different morphological responses as shown in Table 1. The plants were subjected to three watering levels of CSC (100%, 70%, and 50%) and/or single biostimulants (CR, Mob, ND, and NN). Under 100% CSC, NN-treated plants showed the highest leaf number and leaf area and was followed by ND, Mob, and CR. ND and NN treatments at 70% CSC and 50% CSC showed the highest increase in leaf number and leaf area as compared to those of other treatments, as well as those of the control. The tallest plants and highest root dry weight were found treatments with NN. CR and Mob showed no significant differences under 50% CSC. On the other hand, when water stress increased (i.e., 70% CSC and 50% CSC), the values for leaf number (20.19% and 45.77%), leaf area (24% and 52.40%), plant height (17.30% and 41.35%), and root dry weight (15.70% and 49.59%) decreased significantly. Plant fresh and dry weights increased significantly in biostimulant-treated plants compared to those of the control, and the highest increase was for plants subjected to NN followed by ND under different water levels (Table 2). NN-treated plants with 70% CSC water level had the highest IWUE (average of 2.78 kg m^{-3}) in both growth seasons, which was statistically different from that of all other treatments.

3.2. Physiological and Metabolic Performance

Figure 1a–c shows the effects of water stress and biostimulants on gas exchange, namely, A, E, and gs of mint plants during the growing seasons in 2018 and 2019 (shown as averages). Under 100% CSC, the A of mint plants showed a ($p < 0.05$) significant increase in plants subjected to NN (8.35 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) compared to that of other treatments (Figure 1a). The reduction in irrigation water to 70% CSC and 50% CSC caused significantly reduced A values in control plants. However, biostimulant treatment increased the values of A, wherein NN-treated plants under 70% CSC and 50% CSC had the highest values (7.4 and 4.9 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively), followed by ND-treated plants (7.3 and 4.6 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively).

Table 1. Average morphological responses for mint plants grown under greenhouse conditions during the growth season in 2018 and 2019, subjected to three water levels, 100%, 70%, and 50% CSC, and four biostimulants, CR, Mob, ND, and NN, as well as a control (without biostimulants).

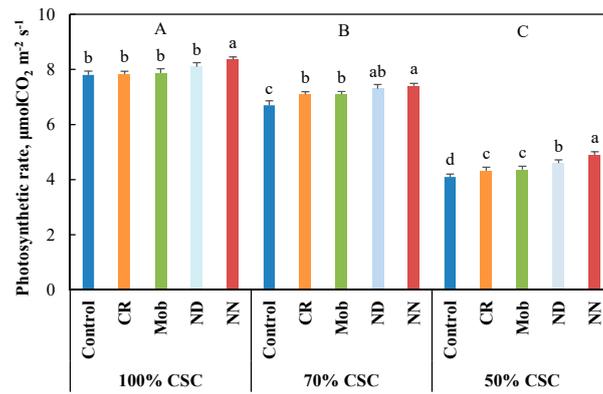
Variable	Leaf Number (plant ⁻¹)						Leaf Area (cm ² plant ⁻¹)						Plant Height (cm)						Root Dry Weight (g)					
	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means
100% CSC	57.00 ^e	62.10 ^d	67.20 ^c	72.30 ^b	77.20 ^a	67.16 ^A	228.00 ^e	247.10 ^d	268.80 ^c	288.20 ^b	307.80 ^a	267.98 ^A	27.60 ^e	29.10 ^d	30.20 ^c	32.10 ^b	35.30 ^a	30.86 ^A	1.05 ^e	1.13 ^d	1.19 ^c	1.29 ^b	1.38 ^a	1.21 ^A
70% CSC	47.00 ^e	51.20 ^d	53.10 ^c	56.80 ^b	59.90 ^a	53.60 ^B	178.60 ^e	193.56 ^d	200.78 ^c	216.80 ^b	228.62 ^a	203.67 ^B	23.10 ^e	24.40 ^d	25.70 ^c	26.90 ^b	27.50 ^a	25.52 ^B	0.82 ^d	1.01 ^c	1.04 ^c	1.12 ^b	1.21 ^a	1.02 ^B
50% CSC	29.00 ^d	34.80 ^c	35.90 ^c	39.30 ^b	43.10 ^a	36.42 ^C	101.50 ^d	121.20 ^c	125.65 ^c	137.55 ^b	151.85 ^a	127.55 ^C	16.20 ^d	17.30 ^c	17.80 ^c	18.90 ^b	20.30 ^a	18.10 ^C	0.56 ^d	0.59 ^c	0.60 ^c	0.64 ^b	0.67 ^a	0.61 ^C
Means	44.33 ^E	49.37 ^D	52.07 ^C	56.13 ^B	60.07 ^A		169.37 ^E	187.29 ^D	198.41 ^C	214.18 ^B	229.42 ^A		22.30 ^E	23.60 ^D	24.57 ^C	25.97 ^B	27.70 ^A		0.81 ^E	0.91 ^D	0.94 ^C	1.02 ^B	1.06 ^A	
Water levels	*** (LSD _{0.05} = 0.877)						*** (LSD _{0.05} = 2.502)						*** (LSD _{0.05} = 0.282)						*** (LSD _{0.05} = 0.010)					
Biosti.	*** (LSD _{0.05} = 1.132)						*** (LSD _{0.05} = 3.230)						*** (LSD _{0.05} = 0.365)						*** (LSD _{0.05} = 0.013)					
Water levels × Biosti.	*** (LSD _{0.05} = 1.961)						*** (LSD _{0.05} = 5.594)						*** (LSD _{0.05} = 0.631)						*** (LSD _{0.05} = 0.022)					

Data represent the mean calculated from $n = 100, 60, \text{ or } 20$ for each water level, biostimulants or their interaction, respectively, in two growth seasons. *** ($p \leq 0.001$). Means differing in lowercase letters (a–e) within each row indicate significant differences at $p \leq 0.05$. Means differing in uppercase letters (A–E) within each mean row and each mean column indicate significant differences according to the least significant difference (LSD) test at $p \leq 0.05$. CSC (container substrate capacity), Control (without biostimulants), CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), NN (Nanozim NXT™), and Biosti. (biostimulant).

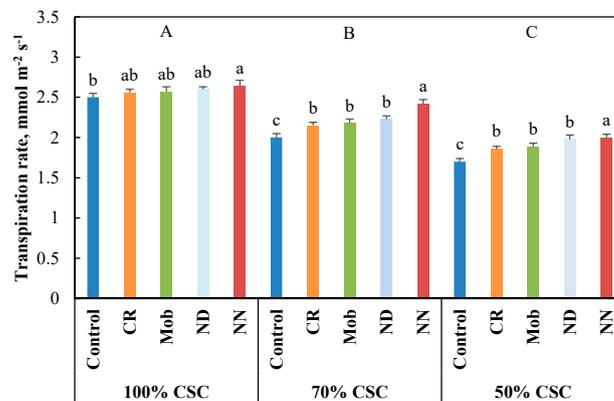
Table 2. Average plant fresh and dry weights and irrigation water-use efficiency (IWUE) for mint plants grown under greenhouse conditions during the growth season in 2018 and 2019, subjected to three water levels, 100%, 70%, and 50% CSC, and four biostimulants, CR, Mob, ND, and NN, as well as a control (without biostimulants).

Variable	Water Applied (mm)	Plant Fresh Weight (g)					Plant Dry Weight (g)					IWUE (kg m ⁻³)							
		Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means
100% CSC	35.91	7.38 ^d	8.01 ^c	8.48 ^b	8.47 ^b	9.24 ^a	8.32 ^A	6.64 ^e	7.05 ^d	7.38 ^c	7.79 ^b	8.41 ^a	7.45 ^A	1.85 ^d	2.01 ^c	2.13 ^b	2.12 ^b	2.32 ^a	2.08 ^B
70% CSC	27.00	6.02 ^d	7.53 ^c	7.39 ^c	7.81 ^b	8.34 ^a	7.42 ^B	5.12 ^d	6.48 ^c	6.50 ^c	6.87 ^b	7.42 ^a	6.48 ^B	2.01 ^d	2.51 ^c	2.46 ^c	2.60 ^b	2.78 ^a	2.47 ^A
50% CSC	22.14	4.69 ^b	4.71 ^b	4.83 ^b	5.18 ^a	5.31 ^a	4.94 ^C	3.75 ^d	3.96 ^c	4.01 ^c	4.25 ^b	4.46 ^a	4.09 ^C	1.91 ^b	1.92 ^b	1.96 ^b	2.11 ^a	2.16 ^a	2.01 ^C
Means		6.03 ^E	6.75 ^D	6.90 ^C	7.15 ^B	7.63 ^A		5.17 ^E	5.83 ^D	5.96 ^C	6.31 ^B	6.76 ^A		1.92 ^E	2.15 ^D	2.18 ^C	2.28 ^B	2.42 ^A	
Water levels		*** (LSD _{0.05} = 0.086)					*** (LSD _{0.05} = 0.075)					*** (LSD _{0.05} = 0.028)							
Biosti.		*** (LSD _{0.05} = 0.111)					*** (LSD _{0.05} = 0.097)					*** (LSD _{0.05} = 0.036)							
Water levels × Biosti.		*** (LSD _{0.05} = 0.193)					*** (LSD _{0.05} = 0.168)					*** (LSD _{0.05} = 0.062)							

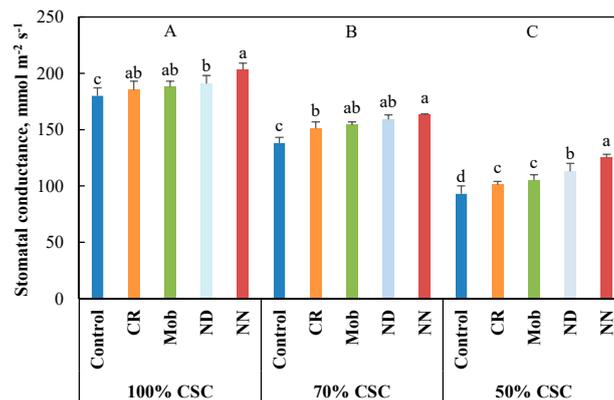
Data represent the mean calculated from $n = 100, 60, \text{ or } 20$ for each water level, biostimulants or their interaction, respectively, in two growth seasons. *** ($p \leq 0.001$). Means differing in lowercase letters (a–e) within each row indicate significant differences at $p \leq 0.05$. Means differing in uppercase letters (A–E) within each mean row and each mean column indicate significant differences according to the least significant difference (LSD) test at $p \leq 0.05$. CSC (container substrate capacity), Control (without biostimulants), CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), NN (Nanozim NXT™), and Biosti. (biostimulant).



(a)

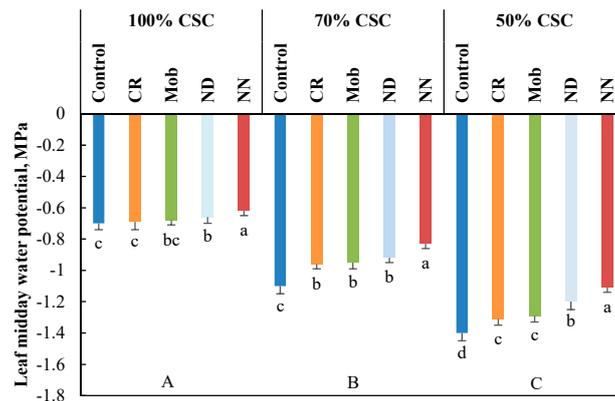


(b)

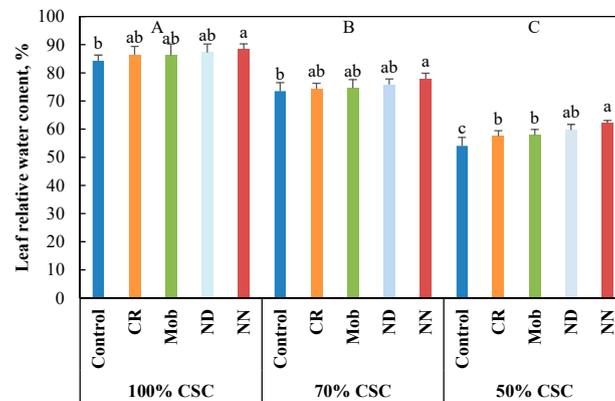


(c)

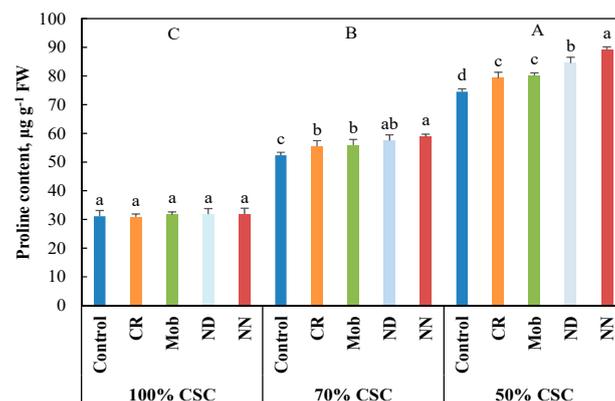
Figure 1. Cont.



(d)



(e)



(f)

Figure 1. Photosynthetic rate (a), transpiration rate (b), stomatal conductance (c), leaf midday water potential (d), leaf relative water content (e), and proline content (f) responses of mint plants, average of two growing seasons, as affected by three water levels, 100%, 70%, and 50% CSC (container substrate capacity), and four biostimulants, CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De’Lite™), and NN (Nanozim NXT™), and a Control (without biostimulants). FW (fresh weight). Different capital letters on top indicate significant differences between water levels at $p \leq 0.05$. Different letters on top of columns indicate significant differences between biostimulants across water levels at $p \leq 0.05$. Bars indicate the means \pm SE of the mean ($n = 20$).

The values of E were higher in plants subjected to biostimulants compared to that of the control (Figure 1b). Treating plants with CR, Mob, and ND showed no significant differences in E under different CSC treatments. However, the E in NN-treated plants was significantly ($p < 0.05$) increased by 5.6%, 21%, and 17.6%, respectively, compared with that of the control plants under 100%, 70%, and 50% CSC.

The values of g_s increased in plants treated with NN (203, 163, and 125 $\text{mmol m}^{-2} \text{s}^{-1}$, respectively) compared to that of other biostimulant treatments under 100%, 70%, and 50% CSC (Figure 1c). Statistically significant differences in g_s under 50% CSC were found only between the ND treatment and both CR and Mob treatments, but significant effects on g_s to the same treatments were not observed for 100% and 70% CSC. Irrespective of the biostimulant treatments, there were significant ($p < 0.05$) differences between the water levels treatments, where the 100% CSC was superior.

Leaf midday water potential increased in plants subjected to different biostimulants under 70% CSC (average of -0.95 MPa) and 50% CSC (average of -1.26 MPa) compared to that of 100% CSC (average of -0.67 MPa), as shown in Figure 1d. The NN treatment had the lowest water potential under different water level treatments. The leaf relative water content increased significantly ($p < 0.05$) in plants subjected to NN under different water levels (Figure 1e), where NN-treated plants with 100% CSC had the highest value of 88.3%. The proline content (Figure 1f) increased in plants subjected stress conditions only (70% and 50% CSC) and the increases were higher in plants subjected to biostimulants than that of the control (without biostimulants) treatment. The NN-treated plants with 50% CSC had the highest proline content value of $89.1 \mu\text{g g}^{-1}$ fresh weight.

3.3. EO Ratio and Constitutes

The EO ratio was increased in response to biostimulant treatments as shown in Figure 2. Control treatments had the lowest EO ratio of 2.7%, 2.8%, and 2.1% fresh weight, respectively, under 100%, 70%, and 50% CSC. In NN-treated plants, the average EO ratio was increased by 21.57%, followed by that of ND (15.81%), in relation to that of the control plants under water level treatments. There was a significant difference in the EO ratio ($p < 0.05$) of mint plants between different biostimulant treatments within each water level, except between CR and Mob ($p > 0.05$) under water stress conditions of 70% and 50% CSC. Irrespective of the biostimulant treatments, the EO ratio under 70% CSC was not significantly higher than that of 100% CSC.

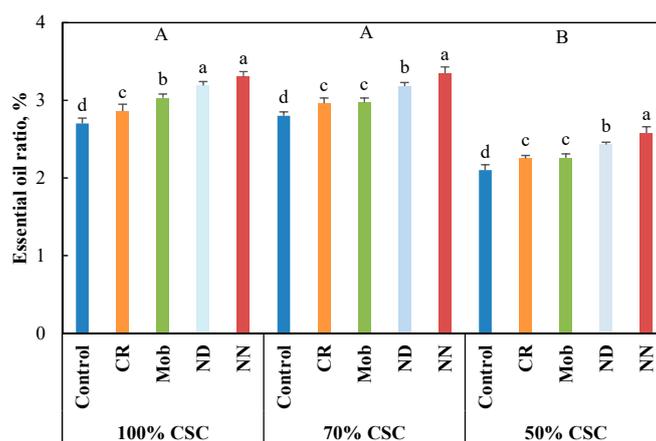


Figure 2. Essential oil ratio of mint plants, average of two growing seasons, as affected by three water levels: 100, 70, and 50% CSC (container substrate capacity), and four biostimulants: CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), and NN (Nanozim NXT™), in addition to Control (without biostimulants). Different capital letters on top are significant differences between water levels at $p \leq 0.05$. Different letter on top columns indicate significant differences between biostimulants across water levels at $p \leq 0.05$. Bars give the means \pm SE of the mean ($n = 20$).

Major EO constituents in all treatments were 1-menthone, isopulegone, pulegone, α -pinene, 1,8-cineol, and α -terpineol ratios as shown in Tables 3 and 4. The 1-menthone, isopulegone and pulegone were significantly ($p < 0.001$) reduced in plants subjected to water stress and biostimulant treatments (Table 3). Irrespective of the biostimulant treatments, 70% and 50% CSC plants exhibited decreased 1-menthone by 5.3% and 9.6%, respectively, compared to that of 100% CSC plants. Likewise, isopulegone for these plants was decreased by 5.3% and 8.6% and pulegone by 6.9% and 8.5%, respectively. Irrespective of the water level treatments, ND and NN treatments significantly reduced 1-menthone, isopulegone, and pulegone, by 23.8% and 33.3%; 8.1% and 18.6%; 11.1% and 18% on average, respectively, compared to that of the control treatment. However, the application of biostimulants significantly ($p < 0.001$) increased the α -pinene, 1,8-cineol, and α -terpineol ratios under different water stress conditions (Table 4). These ratios were significantly ($p < 0.001$) different between 100%, 70%, and 50% CSC plants, where 100% CSC showed the lowest values. The NN-treated plants yielded the highest ratios for α -pinene (4.3%), 1,8-cineol (36.1%), and α -terpineol (3.4%) at 50% CSC.

3.4. Antioxidant Activities

There was a significantly ($p < 0.05$) reduced accumulation of lipid peroxidation and H_2O_2 in plants subjected to different biostimulants, as shown in Figure 3. The NN-treated plants yielded the lowest values of lipid peroxidation (57, 46, and 27 $\mu\text{mol TBARS g}^{-1}$ fresh weight, respectively) and H_2O_2 (2.6, 4.6, and 5.9 $\mu\text{mol g}^{-1}$ fresh weight, respectively) under 100%, 70%, and 50% CSC. However, control plants had the highest values of lipid peroxidation (64, 54, and 37 $\mu\text{mol TBARS g}^{-1}$ fresh weight, respectively) and H_2O_2 (2.9, 5.2, and 7.2 $\mu\text{mol g}^{-1}$ fresh weight, respectively). It was observed that there were no significant differences between the control and CR treatments in lipid peroxidation and H_2O_2 under different water levels, except for the 70% CSC for lipid peroxidation. In 50% CSC, there were only significant differences between the Mob and ND treatments. On the contrary, there were significant ($p < 0.05$) increases in the activities of CAT and SOD of leaf extracts of biostimulant-treated plants under normal and water stress conditions (Figure 3). The highest CAT and SOD activity values were found in NN treatments (increasing 25.5% and 40.3%, respectively), followed by that of ND-treated plants (increasing 17.9% and 26.8%, respectively) comparing with those of the control treatment, which had the lowest values. The CAT activity was significantly ($p < 0.05$) increased by 30% and 58.2% on average, respectively, in plants subjected to water stress (70% and 50% CSC) compared to that of the normal (100% CSC) condition, whereas SOD activity was increased by 79.3% and 123.7% on average, respectively.

Table 3. Average 1-menthone, isopulegone, and pulegone for mint plants grown under greenhouse conditions during the growth season in 2018 and 2019, subjected to three water levels, 100%, 70%, and 50% CSC, and four biostimulants, CR, Mob, ND, and NN, as well as a control (without biostimulants).

Variable	1-Menthone (%)						Isopulegone (%)						Pulegone (%)					
	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means
100% CSC	2.20 ^a	2.10 ^c	1.90 ^c	1.70 ^d	1.50 ^e	1.88 ^A	5.60 ^a	5.50 ^{a,b}	5.40 ^b	5.20 ^c	4.90 ^d	5.32 ^A	56.46 ^a	54.27 ^b	53.51 ^b	50.32 ^c	46.93 ^d	52.30 ^A
70% CSC	2.10 ^a	2.00 ^b	1.80 ^c	1.60 ^d	1.40 ^e	1.78 ^B	5.40 ^a	5.30 ^b	5.20 ^c	5.00 ^d	4.30 ^e	5.04 ^B	53.34 ^a	50.12 ^b	49.68 ^b	47.08 ^c	43.22 ^d	48.69 ^B
50% CSC	2.00 ^a	2.00 ^a	1.70 ^b	1.50 ^c	1.30 ^d	1.70 ^C	5.30 ^a	5.10 ^b	5.00 ^b	4.80 ^c	4.10 ^d	4.86 ^C	52.13 ^a	49.52 ^b	48.31 ^c	46.52 ^d	42.71 ^e	47.84 ^C
Means	2.10 ^A	2.04 ^B	1.80 ^C	1.60 ^D	1.40 ^E		5.44 ^A	5.30 ^B	5.20 ^C	5.00 ^D	4.43 ^E		53.98 ^A	51.30 ^B	50.50 ^C	47.98 ^D	44.29 ^E	
Water levels	*** (LSD _{0.05} = 0.016)						*** (LSD _{0.05} = 0.046)						*** (LSD _{0.05} = 0.545)					
Biosti.	*** (LSD _{0.05} = 0.021)						*** (LSD _{0.05} = 0.059)						*** (LSD _{0.05} = 0.703)					
Water levels × Biosti.	*** (LSD _{0.05} = 0.036)						*** (LSD _{0.05} = 0.102)						ns					

Data represent the mean calculated from $n = 100, 60, \text{ or } 20$ for each water level, biostimulants or their interaction, respectively, in two growth seasons. ^{ns} (not significant) and ^{***} ($p \leq 0.001$). Means differing in lowercase letters (a–e) within each row indicate significant differences at $p \leq 0.05$. Means differing in uppercase letters (A–E) within each mean row and each mean column indicate significant differences according to the least significant difference (LSD) test at $p \leq 0.05$. CSC (container substrate capacity), Control (without biostimulants), CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), NN (Nanozim NXT™), and Biosti. (biostimulant).

Table 4. Average α -pinene, 1,8-cineol, and α - terpineol ratios of mint plants grown under greenhouse conditions during the growth season in 2018 and 2019, subjected to three water levels, 100%, 70%, and 50% CSC, and four biostimulants, CR, Mob, ND, and NN, as well as a control (without biostimulants).

Variable	α -Pinene (%)						1,8-Cineol (%)						α - Terpineol (%)					
	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means	Control	CR	Mob	ND	NN	Means
100% CSC	0.90 ^e	1.80 ^d	3.40 ^c	3.60 ^b	4.00 ^a	2.74 ^C	24.28 ^c	25.09 ^c	25.20 ^c	27.31 ^b	29.11 ^a	26.20 ^A	1.70 ^e	1.90 ^d	2.30 ^c	2.50 ^b	2.70 ^a	2.22 ^C
70% CSC	1.50 ^e	2.50 ^d	3.80 ^b	3.70 ^c	4.20 ^a	3.14 ^B	25.62 ^c	26.62 ^c	26.70 ^c	28.61 ^b	35.71 ^a	28.65 ^B	1.90 ^e	2.10 ^d	2.40 ^c	2.70 ^b	3.10 ^a	2.44 ^B
50% CSC	2.10 ^e	3.10 ^d	3.70 ^c	4.10 ^b	4.30 ^a	3.46 ^A	26.82 ^c	27.79 ^c	27.92 ^c	29.82 ^b	36.11 ^a	29.70 ^C	2.10 ^e	2.30 ^d	2.50 ^c	3.00 ^b	3.40 ^a	2.66 ^A
Means	1.50 ^E	2.47 ^D	3.63 ^C	3.80 ^B	4.17 ^A		25.57 ^D	26.50 ^C	26.61 ^C	28.58 ^B	33.64 ^A		1.90 ^E	2.10 ^D	2.40 ^C	2.73 ^B	3.07 ^A	
Water levels	*** (LSD _{0.05} = 0.047)						*** (LSD _{0.05} = 0.544)						*** (LSD _{0.05} = 0.018)					
Biosti.	*** (LSD _{0.05} = 0.061)						*** (LSD _{0.05} = 0.702)						*** (LSD _{0.05} = 0.024)					
Water levels × Biosti.	*** (LSD _{0.05} = 0.105)						*** (LSD _{0.05} = 1.216)						*** (LSD _{0.05} = 0.041)					

Data represent the mean calculated from $n = 100, 60, \text{ or } 20$ for each water level, biostimulants or their interaction, respectively, in two growth seasons. ^{***} ($p \leq 0.001$). Means differing in lowercase letters (a–e) within each row indicate significant differences at $p \leq 0.05$. Means differing in uppercase letters (A–E) within each mean row and each mean column indicate significant differences according to the least significant difference (LSD) test at $p \leq 0.05$. CSC (container substrate capacity), Control (without biostimulants), CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), NN (Nanozim NXT™), and Biosti. (biostimulant).

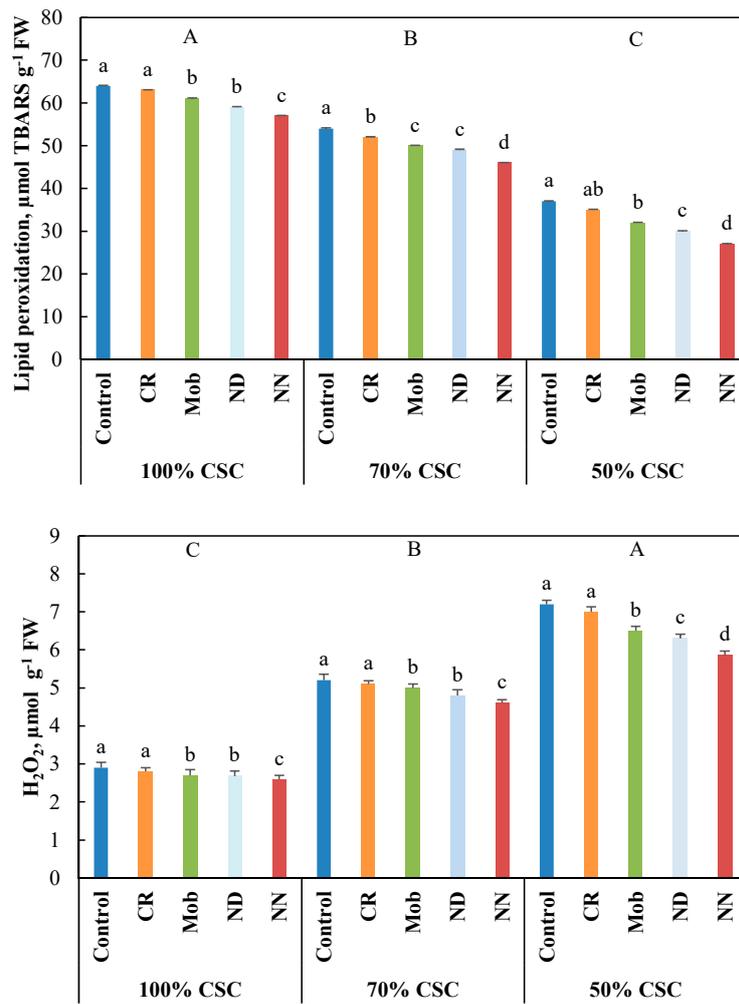


Figure 3. Cont.

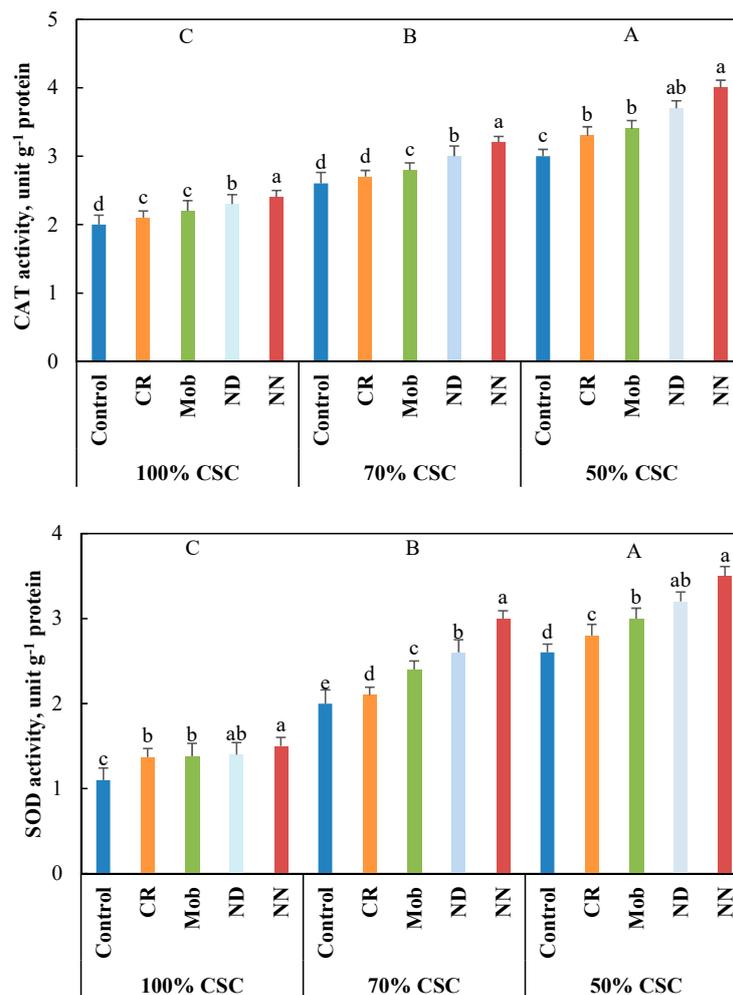


Figure 3. Lipid peroxidation, H₂O₂, catalase (CAT), and superoxide dismutase (SOD) activities of mint plants, average of two growing seasons, as affected by three water levels, 100%, 70%, and 50% CSC (container substrate capacity), and four biostimulants, CR (CRADLE™), Mob (Mobilizer™), ND (Nanozim De'Lite™), and NN (Nanozim NXT™), and a control (without biostimulants). FW (fresh weight). Different capital letters on top indicate significant differences between water levels at $p \leq 0.05$. Different letters on top columns indicate significant differences between biostimulants across water levels at $p \leq 0.05$. Bars provide the means \pm SE of the mean ($n = 20$).

4. Discussion

Water stress is one of the major limiting factors of the growth and productivity of plants worldwide [21]. The amount of irrigation water applied influenced the biomass and EO yields of mint. The fresh and dry weights of mint were decreased with the irrigation water stress because of vegetative growth (i.e., leaf number and plant height), which decreased under water deficit conditions. Reduction in growth parameters as a consequence of drought has also been described in peppermint [6,7,11], Japanese mint [9], purple basil [8], balm [10], and chamomile [12]. The irrigation water level of 50% CSC had a negative effect on EO yield of mint. This is in agreement with earlier findings in peppermint [11] and chamomile [12], and in contrary to the results found in the previous studies from Ekren et al. [8] in purple basil and Farahani et al. [10] in balm.

The application of biostimulants under water stress conditions (70% and 50% CSC) showed enhanced growth by means of increased leaf number, plant height, root dry weight, fresh and dry weights, and IWUE. These morphological improvements are mainly attributed to the composition of these biostimulants. The most active biostimulant in this study was NN, which is composed of a unique

mixture of important compounds: seaweed extract (15%), humic acid (5%), macro (potassium 1% and phosphorus) and micro elements, alginic acid, and hydrolyzed protein, as described in the materials and methods. The major constituents of the NN biostimulant were seaweed extracts (*Ascophyllum nodosum*) and humic acid. The application of *Ascophyllum nodosum* extracts as plant biostimulants has been reported in several studies [19,22]. Humic acid may increase the leaf area, stem diameter, plant dry weight in different plants [23,24] and may ameliorate stress conditions in tomatoes [25]. Potassium, phosphorus, and microelements play critical roles in the growth and morphology of most plants [26,27]. However, the mixture was superior in the ameliorating effects against water stress in mint plants compared to other commercial biostimulants. The second biostimulant showing relatively high morphological performance was ND, which is mainly composed of *Ascophyllum nodosum* extracts (25%), carbohydrates (25%), (*w/w*) amino acid (2%) and potassium (1%). ND showed slightly lower morphological promoting effects than that of NN. CR and Mob are mainly composed of mycorrhizal biofertilizer. However, Mob contains additional components, including seaweed extract (*Macrocystis pyrifera*), humic acid, and amino acids, which may explain the slight increased vegetative performance of Mob compared to that of CR. Furthermore, *Macrocystis pyrifera* has been reported to have stimulatory effects on plant growth [28].

Gas exchange parameters (g_s , E , and A) are important indicators of the physiological performance of plants under stress conditions. The increase in g_s under stress conditions in response to external factors is strongly related to enhanced gas exchange through the stomata [29]. The increased gas exchange is normally reflected as enhanced transpiration and photosynthesis rates in the leaves [30]. There were increases in the gas exchange in plants treated with different biostimulants under water stress conditions, which indicated that these biostimulants acted as effective stress ameliorants. Leaf water potential and relative water content reductions might be associated with stress conditions [31,32]. They increased in this study in plants subjected to different biostimulants, indicating enhanced metabolic performance of treated plants. Furthermore, the increased proline composition in biostimulant-treated plants reflected enhanced stress tolerance as found in previous studies using other external elicitors [22,33].

The main constituents of the EO were pulegone and 1,8-cineol. A previous study on the same species from Egypt reported comparable composition of both compounds [1]. There were fluctuations in the main constituents of EO, as well as specific compounds, including pulegone, isopulegone, 1-menthone, 1,8-cineol, α -pinene, and α -terpineol. Secondary metabolites, such as cineole are usually associated with terpenes [34] and this explains the parallel increase in 1,8-cineol, α -terpineol, and pinene. 1-menthone and isopulegone are metabolites of pulegone. The pulegone is not favored in the EO composition of mint plants because of its carcinogenic effects at high doses [1], whereas, cineol is a favored compound in EOs because of its medicinal applications and pharmaceutical potential [35,36]. The application of NN showed the highest increase in 1,8-cineol and related terpenes ratios and lowest compositions of pulegone compared to that of the control and other biostimulant treatments. This result suggests that NN application may have future applications in medicinal plants, such as mints. The use of the NN biostimulant is a novel approach for enhancing the chemical composition of the EOs of mint plants by reducing hazardous compounds and increasing useful ones as found in this study.

In this study, the application of seaweed extract-based biostimulants mixed with humic acid and/or macro elements represented a novel tool for the enhancement of the medicinal properties of major medicinal plants, such as mints. The achievement of enhanced antioxidant activities of the EOs of mint might be of great importance for agricultural and related pharmaceutical industries. The oil of mints is routinely used in perfume and cosmetic preparations, as well as in the food industries, such as in chocolate and soft drinks. The development of new EO compositions with increased antioxidant properties will increase the additive value of the medicinal crop and will assist in reducing dependence on synthetic antioxidants to control ROS accumulation.

5. Conclusions

This study revealed an association between the application of specific biostimulants and the increase/decrease of the main EO composition (cineol and pulegone) of mint plants. The application of this finding is related to the agricultural, medicinal, and pharmaceutical industries. There were increases in the morphological characteristics, physiological performance, and EO ratio of biostimulant-treated plants. The morphological and physiological enhancements indicated increased tolerance to water stress. Further, biostimulant-treated plants showed higher antioxidant activities, reduced accumulation of H₂O₂, and increased CAT and SOD activities, which indicated an antioxidant stress tolerance activation mechanism in treated plants. The application of biostimulants to mint plants increased the quantity and quality of produced EOs and enhanced the medicinal properties, as well as that of the traditional medicinal crop. ND and NN are recommended under water stress conditions in mint.

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