Melatonin-mediated regulation of growth, physiological, biochemical, and essential oil constituents alleviates drought stress in oregano (Origanum vulgare L.)

Hossein Gorgini Shabankareh
Post-doctoral researcher of Medicinal, Tarbiat Modares University (TMU)
Mohammad Reza Asgharipour
m_asgharipour@uoz.ac.ir

University of Zabol
Ali Salehi Sardoei
South Kerman Agricultural and Natural Resources Research and Education Center, AREEO
Fatemeh Gorgini Shabankareh
University of Shiraz

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Abstract

Drought stress reduces oregano growth, yield, and yield components, significantly limiting global agricultural productivity. This study aimed to investigate the effects of drought stress and the foliar application of melatonin on the antioxidant properties and secondary metabolites of *Origanum vulgare* L. sp. vulgare during the 2023 growing season. Drought stress was imposed by withholding irrigation at four levels: control (90–100% readily available water, RAW), 70–80%, 50–60%, and 30–40% RAW. Melatonin was applied foliarly at three concentrations: 0, 100, and 200 mM, during the vegetative growth completion, flowering initiation, and full flowering stages, suggesting its potential as a sustainable tool for enhancing drought tolerance in other crops, with future research directed toward elucidating its molecular mechanisms. Gas chromatography-mass spectrometry (GC/MS) was utilized to identify and analyze the components of the essential oil. The ANOVA results demonstrated that both growth and phytochemical traits were significantly affected by the individual treatments and their interactions. Exposure to different levels of melatonin during drought stress helped mitigate the adverse effects of stress on dry mass and essential oil yield compared to the control group. However, under drought stress, proline content, total phenols, total flavonoids, soluble carbohydrates, antioxidant enzyme activities, and essential oil yield all increased. Interaction results indicated that while drought stress combined with melatonin application (especially at 200 mM) reduced antioxidant activity compared to the control group, certain essential oil components were significantly altered under various experimental treatments. Specifically, after drought stress, only the content of linalool, terpinolene, and caryophyllene increased in plants that had received the 200 mM melatonin foliar application compared to other drought levels. In conclusion, the combined use of melatonin and re-watering after drought stress appears to be a promising and environmentally friendly strategy to enhance drought tolerance, growth, and phytochemical content in oregano plants.

1. Introduction

Environmental stresses significantly threaten global agricultural productivity, with drought being a particularly severe factor [1]. Different plant species show varying physiological and morphological responses to drought conditions. Drought can damage the photosynthetic apparatus, which leads to reduced plant metabolism, growth, and yield. To survive and thrive in water-limited environments, plants typically adopt either drought escape or drought tolerance strategies [2]. Water scarcity is a major contributor to reduced yields in agricultural crops, including horticultural plants, with the extent of yield loss depending on the plant's response to stress [3]. Mitigating the harmful effects of drought or enhancing plant tolerance to these conditions can be crucial for overcoming production constraints in arid and semi-arid regions [4].

Drought poses a significant challenge to Origanum cultivation worldwide, particularly in Mediterranean regions where the genus is native and widely grown for its culinary and medicinal value. Globally, water scarcity reduces oregano yields by limiting plant growth and essential oil production, threatening the economic viability of this crop in arid and semi-arid climates such as parts of southern Europe, North Africa, and the Middle East [5]. In Iran, a country within the Irano-Turanian phytogeographical region where Origanum vulgare subspecies like viride, vulgare, and gracile are distributed, drought exacerbates these challenges, especially in the northern, northwestern, and western provinces. Increasing aridification, driven by climate change and irregular precipitation patterns, has led to reduced biomass and quality of oregano harvests, impacting local farmers and the medicinal plant industry [6]. This study's focus on drought tolerance in oregano is thus critical for addressing these pressing agricultural concerns both globally and locally.

Melatonin is an indoleamine derived from tryptophan that has recently gained attention for its potential use in enhancing plant resistance to various stresses and improving crop yield and quality in field conditions. This natural compound is found throughout plants, including in roots, stems, leaves, flowers, fruits, and seeds [7]. Initially isolated from bovine pineal glands and identified as N-acetyl-5-methoxytryptamine [8], melatonin is classified as a phytohormone. Melatonin plays a crucial role in numerous physiological processes, including growth regulation, increased photosynthetic efficiency, improved defense mechanisms, modulation of stress-responsive gene expression, and enhanced tolerance to abiotic stresses [9]. Furthermore, it possesses strong antioxidant properties, effectively scavenging free radicals [10]. As an amphipathic molecule, melatonin can easily cross cell membranes, allowing for its distribution within cells. In vascular plants, melatonin serves various functions, particularly as an antioxidant that protects against both biotic and abiotic stresses [11]. Additionally, research has shown that melatonin can regulate plant growth and enhance crop yield [12].

Melatonin serves several important functions in plants, acting as a messenger of darkness and a growth regulator. It plays a crucial role as an antioxidant, helping to protect plants from both internal and environmental oxidative stresses [13, 14, 15]. Studies have demonstrated that the foliar application of melatonin at different concentrations can increase the levels of soluble carbohydrates and Pro [16]. This treatment also enhances the relative water content (RWC) in leaves [17], ultimately boosting plant metabolism and enabling the plants to better withstand stressful conditions [18]. Research on melatonin's role in stress response has uncovered a complex relationship involving the production of reactive oxygen species (ROS) [19]. Evidence indicates that melatonin functions as a broad-spectrum antioxidant, capable of inactivating or preventing the formation of these reactive species [10].

Oregano (*Origanum* sp.) is a perennial herb that belongs to the Lamiaceae family. This genus includes numerous species, primarily found in the wild in Mediterranean regions, and it exhibits significant morphological and chemical diversity worldwide [5]. One of the most important species in this genus is *O. vulgare* L., which has a wide distribution not only in the Mediterranean but also in other parts of the world, including the Irano-Turanian region. This species contains six subspecies globally [20], of which only three—viride, vulgare, and gracile—have been identified in Iran, distributed across the north, northwest, and west regions, respectively [6]. The vegetative body of this plant contains essential oil, primarily composed of phenolic monoterpenes, especially carvacrol, along with flavonoids, bitter substances, and mucilage compounds. Various oregano species are widely recognized as popular spices around the world. In addition to their culinary uses, the active compounds in oregano are employed in traditional medicine to treat coughs, serve as an expectorant, carminative, appetite stimulant, diuretic, stomach tonic, and sedative. Due to its rich content of phenolic compounds, the essential oil of oregano possesses strong antibacterial, antifungal, and antioxidant properties [21, 6].

This study focuses on the effects of drought stress on the physiological and biochemical characteristics, as well as the yield, of oregano (*Origanum vulgare* L. sp. vulgare). It also examines the role of melatonin in enhancing plant resistance to drought stress and improving growth traits. The aim was to investigate

how melatonin affects the essential oil yield, antioxidant properties, and secondary metabolite profile of oregano under water stress conditions.

We hypothesized that foliar application of melatonin would mitigate the adverse effects of drought stress on Origanum vulgare by enhancing antioxidant enzyme activity, increasing osmoprotectant accumulation, and improving essential oil yield. Specifically, we predicted that higher melatonin concentrations (e.g., 200 mM) would result in greater drought tolerance compared to untreated plants, as evidenced by improved growth parameters and phytochemical content under water-limited conditions.

2. Materials and Methods

2.1 Plant Materials

This experiment was conducted at the Deland Agricultural Research Unit (Model Farm), Gorgan, Iran (36°30' N, 53°57' E, 155 m above sea level) during the 2022–2023 growing season. A factorial experiment based on a randomized complete block design with three replications (each replication consisted of three experimental units) was employed to investigate the effects of two factors: irrigation regimes and melatonin on the medicinal herb oregano (*Origanum vulgare* L. sp. vulgare). Irrigation regimes were set at four levels: control (90–100% readily available water, RAW), 70–80%, 50–60%, and 30–40% RAW.

Melatonin, obtained from Sigma-Aldrich, was foliarly applied at three concentrations—0, 100, and 200 µM—across three growth stages (vegetative growth completion, flowering initiation, and full flowering), with applications conducted in the early morning (between 6:00 and 8:00 AM) to optimize absorption. The selected concentrations were based on previous reports and preliminary tests conducted for this plant [22].

Oregano seeds were obtained from Strictly Medicinal Seeds, USA. To break seed dormancy, seeds were placed in a completely moist environment at 4°C for three weeks in May. In June, the seeds were sown in seed trays (with an equal ratio of cocopeat and perlite). Approximately three months after sowing in the seed trays (September), seedlings were transplanted into individual pots (6 cm diameter and 10 cm height) to produce seedlings. After about four months, in January, when the seedlings reached the 10-leaf stage, they were transplanted into larger pots with a diameter of 30 cm and a height of 40 cm (containing two parts soil, one part perlite, and one part leaf mold). A total of 108 plants (including sub-units in each replication) were used for cultivation.

2.2 Treatments and Experimental Setup

Drought stress treatments were applied using a gravimetric method. Equal amounts of gravel were placed at the bottom of each pot for drainage, and the pots were filled with soil to a uniform weight (9 kg per pot). The soil in each pot was saturated with water and allowed to drain for 48 hours on a mesh platform to reach field capacity. The pots were then weighed, and the soil was completely dried at 105°C for 48 hours to determine the dry weight. The percentage of soil moisture at field capacity was determined, and the amount of water required for each drought treatment was calculated by weighing a sample pot from each block. To control for the dry weight of the plants, each drought treatment included an additional pot to ensure that the dry weight of the plants was not included in the calculated water requirements. For the first two months after transplanting the main pots to the field (mid-June), the pots were irrigated under the same regime. From this point onward (late March, approximately 9 months after sowing), daily soil moisture measurements were taken to determine irrigation requirements. Irrigation was applied to each treatment when the soil moisture percentage fell below the desired level [23].

To prepare the melatonin solutions, the compound was dissolved in 0.5 mL of 1N sodium hydroxide solution and then diluted with distilled water to achieve the desired concentrations before being applied as a foliar spray. To characterize the soil properties, soil samples were collected and analyzed in the laboratory. The results of the soil analysis are presented in Table 1.

2.3 Essential Oil Extraction

The essential oil from the plant under study was extracted using a Clevenger apparatus with distilled water over a three-hour period. Specifically, 100 grams of dried plant material was placed in a flask and 900 mL of sterile distilled water was added. The extracted essential oil was dried over anhydrous sodium sulfate, stored in dark glass vials at 4°C, and kept in a refrigerator until analysis and biological assays [24].

2.4 Essential Oil Component Identification

Identification and analysis of the essential oil components, after drying with anhydrous sodium sulfate, were performed by analyzing the mass spectra obtained from an Agilent gas chromatography-mass spectrometry (GC/MS) system (MS 5975C, GC7890). These parameters were compared with those of standard compounds. The system was equipped with an HP-5MS column, 30 meters in length and 0.25 mm in diameter. The ionization mode was EI with an ionization energy of 70 electron volts. The oven temperature program was set at 50°C for 5 minutes, followed by a ramp of 3°C/min to 240°C and held for 15 minutes, and finally a ramp of 15°C/min to 300°C. The injector temperature was set at 290°C, using helium as the carrier gas at a flow rate of 0.8 mL/min. Spectrum identification was performed by comparing the components with standard compounds using retention indices (RI) and information from reliable scientific sources [25].

2.5 Trait Measurement

Approximately 10 weeks after the initiation of the irrigation treatments (when 50% of the plants were in full flowering stage), measurements were taken for growth (dry weight), physiological (RWC), and biochemical parameters (proline, antioxidant activity, total phenols, total flavonoids, soluble carbohydrates, catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD)). Three samples were collected from each treatment for analysis. To determine the dry weight of the aerial parts, the shoots (excluding roots) were harvested, placed in a forced-air oven at 70°C for 72 hours to remove all moisture, and then weighed using a precision balance. The initial mention of drying at room temperature was erroneous and has been corrected; all dry weight measurements were consistently obtained after oven-drying at 70°C.

A methanolic extract was prepared from the dried aerial parts to assess antioxidant-related parameters. Specifically, 0.5 g of oven-dried (70°C for 72 hours) aerial shoot material from each replicate was ground into a fine powder, mixed with 10 mL of 80% methanol, and agitated on a shaker at 150 rpm for 24 hours at room temperature to extract soluble compounds. The mixture was then filtered through Whatman No. 1 filter paper, and the resulting methanolic extract was used to measure parameters such as total antioxidant activity, total phenols, total flavonoids, soluble carbohydrates, leaf proline, and antioxidant enzymes. Total antioxidant activity was quantified using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, as described in the subsequent subsection, while total phenols and flavonoids were determined spectrophotometrically using the Folin-Ciocalteu and aluminum chloride methods, respectively. Antioxidant enzyme activities (catalase, peroxidase, ascorbate peroxidase, and superoxide dismutase) were assessed from the same extract following enzyme-specific protocols outlined below. The method of Bates et al. (1973) was used for Pro determination [26].

Table 1. Physicochemical Properties of Potting Media

Description	Depth (cm)	pH	EC*10 (ds/m)	SP	N (%)	O.C %	P(av) ppm	K(av) ppm	Clay %	Silt %	Sand %	Texture
Deland (Gorgan)	0–30	7.34	4.076	141.86	0.09	0.9	24.8	256	16	42	42	Clay-Silty

2.6 DPPH Radical Scavenging Activity

To determine the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, 1 mL of methanolic extract was mixed with 1 mL of 0.1 mM DPPH solution. For the control, 1 mL of pure methanol was substituted for 1 mL of methanolic extract, and pure methanol was used as a blank. After 30 minutes in the dark, the samples were read at 517 nm using a spectrophotometer. The obtained absorbance values were converted to percentage of radical scavenging activity using Eq. 1 [27].

DPPH scavenging (%) = ((Absorbance of control - Absorbance of sample) / Absorbance of control) × 100 Eq. 1

The obtained values represent the percentage of DPPH radical scavenging activity in the methanolic extract (10 ppm) of the samples [28].

2.7 Catalase (CAT) Activity

CAT activity was measured according to the method of Maehly and Chance (1954), based on the decomposition of hydrogen peroxide by CAT [29]. The reaction mixture contained 3 mL of 50 mM sodium phosphate buffer (pH 7) containing 51.4 µM hydrogen peroxide and 50 µL of extract. After adding the extract, the decrease in absorbance at 240 nm was measured for 1 minute using a spectrophotometer.

2.8 Peroxidase (POD) Activity

POD activity was assayed according to Herzog and Fahimi (1976) [30]. The reaction mixture contained 3 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 51.4 µM hydrogen peroxide, 35.3 µL of guaiacol, and 50 µL of extract. After adding the extract, the increase in absorbance at 470 nm was measured for 2 minutes using a spectrophotometer.

2.9 Ascorbate Peroxidase (APX) Activity

APX activity was determined according to Nakano and Asada (1981) [31]. 0.2 g of frozen plant sample was ground with 3 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 5 mM EDTA, 5 mM dithiothreitol, 100 mM NaCl, and 2% (w/v) polyvinylpyrrolidone in a mortar on ice. The extract was filtered through filter paper. The resulting homogenates were centrifuged at 15,000 rpm for 15 minutes at 4°C, and the supernatant was used to assay APX activity. The reaction mixture contained 50 mM sodium phosphate buffer, 44 µM hydrogen peroxide, 5 mM ascorbate, and enzyme extract. The decrease in absorbance of ascorbate at 290 nm was determined and expressed per mg protein in the enzyme extract.

2.10 Superoxide Dismutase (SOD) Activity

SOD activity was measured according to Giannopolitis and Ries (1977) [32]. The reaction mixture, in a final volume of 1 mL for the assay of SOD activity, contained 835 µL of 50 mM sodium phosphate buffer (pH 7.8), 50 µM nitroblue tetrazolium (NBT), 13 µM riboflavin, 75 nM EDTA, and 50 µL of enzyme extract. The changes in absorbance of the reaction mixture were measured against a blank at 560 nm using a spectrophotometer, and SOD activity was expressed as µmol/min/mg protein.

2.11 Statistical Analysis

Descriptive statistical analysis was initially conducted to understand the quality of the collected data. Descriptive statistics serve to simplify and summarize large datasets for easier interpretation. Subsequently, more complex analyses were performed. Duncan's test, implemented using SPSS software (version 26; IBM Corp., Armonk, NY, USA), was employed to calculate mean squares and experimental errors to determine differences between datasets. Duncan's multiple range test (DMRT) at a significance level of $p \leq 0.05$ was used to identify significant differences among means. Pearson correlation coefficient was calculated using SPSS (version 26; IBM Corp., Armonk, NY, USA) to assess the correlation between different pairs of parameters. Furthermore, stepwise regression analysis at $p < 0.01$ was employed to identify key features influencing oregano dry mass. Path analysis was conducted to determine the direct and indirect effects of each feature in the model (based on the attributes that best explained the variation). Cluster analysis was employed to categorize traits for category, aiming to promote physiological parameters. All the above calculations (normality of data distribution, simple correlation coefficient, stepwise regression, and path analysis) were performed using SPSS ver. 26, and the causal diagram was drawn using AMOS ver. 24.

3. Results

3.1 Essential Oil Composition

GC-MS analysis of the treatments revealed that the secondary compounds of oregano varied between 93.97% and 83.95%. The most abundant compounds were terpinolene, Caryophyllene, and germacrene D (Table 2). Comparing the effects of stress and melatonin treatment showed that their application under 40% stress + 200 mM melatonin conditions led to an increase in secondary compounds compared to other treatments. Comparing the application levels of this hormone indicated that increasing the application rate also resulted in an increase in most of the studied traits (Table 2). This suggests a potential role of this hormone in increasing secondary compounds.

Table 2. Compounds identified in oregano (*Origanum vulgare* L.) essential oil in response to drought stress and melatonin

NO		Compound	RI	Area%											
				D1M1	D1M2	D1M3	D2M1	D2M2	D2M3	D3M1	D3M2	D3M3	D4M1	D4M2	D4M3
1	α-Thujene	926	1.1	1	0.5	0.9	0.76	0.67	0.61	0.85	1.00	0.72	0.98	1	
2	α-pinene	936	1.18	1.04	1	2.19	1.04	1	0.78	0.92	1.10	1.22	1.37	1.24	
3	Camphene	954	1.1	0.82	0.78	2	0.82	0.45	1.11	1.27	0.23	0.16	0.16	0.12	
4	Sabinene	975	5.76	6.21	6.32	5.25	7.24	8.11	5.41	6.39	9.10	6.92	8.31	7.21	
5	β-Pinene	979	0.17	0.51	0.81	0.2	0.23	0.76	1.33	0.25	0.62	0.15	0.10	0.11	
6	Octen-3-ol	983	1.91	2.12	2.80	2.76	1.08	1	2.83	1.45	1.78	1.98	1.11	1.52	
7	Myrecene	991	1.31	0.4	0.7	1.45	1.14	0.96	2.10	1.12	1.33	0.23	0.56	0.4	
8	Octanol	998	0.09	0.49	0.56	0.17	0.46	0.52	1	1.24	0.19	0.67	0.24	0.19	
9	α-Terpinene	1017	5.77	5.41	4.98	4.14	6.78	6.91	4.79	7.21	8.43	6.41	6.90	7.32	
10	Limonene	1032	0.23	1.21	2.22	1.29	0.88	1.56	3.00	3.10	4.11	1.78	1.91	2.21	
11	Carene	1030	0.09	1.03	1.27	1.35	0.90	1.33	0.61	1	1.42	1	1.45	1.03	
12	Ocimene	1038	0.1	2.52	2.91	0.18	3.41	2.12	1.46	1.98	2.90	2.34	2.87	2.97	
13	Linalool	1102	5.16	5.42	7.34	3.45	6.77	6.81	4.71	6.32	6.80	6.98	7.16	8.42	
14	Terpinolen	1182	16.67	16.90	17.98	16.45	16.67	17.10	15.90	16.61	16.90	18.10	23	23.74	
15	Geraniol	1253	0.19	1.55	3.21	1.1	1.17	1.98	2.70	1.74	1.19	3.21	2.42	0.55	
16	Caryophyllene	1419	17.78	14.22	14	16.2	13.19	13	10.43	14.21	16.10	11.24	13.20	16.22	
17	Farensene	1506	1.44	6.72	6.94	3.21	4.90	5.99	3.21	3.99	6.10	5.98	8.61	9.77	
18	Gemacrene D	1487	19.11	14.71	12	16.5	16	10.22	11.21	8.45	8.21	7.66	7.73	9.71	
19	α-Muurolene	1480	1.19	-	-	0.1	0.54	0.82	2.24	2.97	0.90	2.12	1.23	-	
20	Bisabolene	1539	0.55	0.27	0.38	0.33	0.45	0.49	3.11	0.87	0.34	1.42	1	0.23	
21	Caryophyllene oxide	1583	7.19	6.97	5.32	7.1	5.43	5.11	5.41	5.10	4.19	4.69	4.10	3.97	
Total			88.09	89.52	91.95	86.32	89.86	86.91	83.95	87.04	92.94	84.98	94.41	97.93	

RAW (%): D1: 100, D2: 80, D3: 60, D4: 40 FC. Melatonin (mM): M1: 0, M2: 100, M3: 200 mM.

3.2 Cluster Analysis Results

Cluster analysis was performed to group all the secondary compounds of the oregano medicinal plant (Fig. 2). The dendrogram resulting from the cluster analysis of the secondary compounds of the oregano medicinal plant showed that the 21 studied secondary compounds of the oregano medicinal plant were divided into two distinct groups (Fig. 2). In the first group obtained from the cluster analysis, two separate branches were distinguished. In the first sub-branch, one compound (Terpinolene) was placed, and in the second sub-branch, two compounds (Caryophyllene and Germacrene D) were separated. The second group was also divided into two separate branches. In the first branch, five compounds (Sabinene, α-Terpinene, linalool, farnesene, and Caryophyllene oxide) were placed, and the remaining compounds were placed in the second sub-branch, indicating a significant difference between these compounds and the other studied compounds based on phytochemical properties.

3.3 Descriptive Statistical Analysis of Biochemical Traits

Descriptive statistics, including mean, standard deviation, minimum, maximum, range, and coefficient of phenotypic variation, for various traits are summarized in Table 3. Among the descriptive statistics, the coefficient of phenotypic variation for SOD (82.4%) and POD (70.4%) enzyme activities exhibited the highest percentage among the studied traits, while the soluble carbohydrate index, with a value of 53.2, had the lowest percentage of variation.

Examination of Table 3 reveals that CAT, POD, and SOD traits displayed the widest range of variation and the highest standard error. The results of this experiment demonstrated considerable variation among the evaluated physicochemical traits (Table 3).

Table 3
Descriptive statistics related to the various traits in oregano plants at first year

Trait	Mean ± SE	PCV (%)	Minimum	Maximum	Range
RWC	0.27 ± 0.011	4.04	0.18	0.44	0.26
Antioxidant activity	55.80 ± 3.141	3	11	87.11	76.11
Proline	2.47 ± 0.166	2.51	0.92	4.9	3.98
Soluble sugar	46.83 ± 3.126	2.53	2.16	96.23	94.07
Dry mass	20.37 ± 1.107	3.11	13.03	33.98	20.94
Phenol total	1.27 ± 0.081	2.65	0.54	2.37	1.82
Flavonoid total	0.37 ± 0.015	4.01	0.16	0.65	0.49
Essential oil rate	1.42 ± 0.054	4.45	1.14	2.16	1.02
EO yield	0.27 ± 0.011	4.04	0.18	0.44	0.26
SOD	288.44 ± 10.104	4.82	195.44	464.38	268.94
CAT	297.23 ± 14.942	3.36	148.44	546.68	398.24
POD	221 ± 7.950	4.70	75.26	368.01	292.75
AXP	213.07 ± 9.902	3.63	18.1	359.89	341.79

3.4 Comparison of deficit irrigation and Melatonin mean

A comparison of means for the stress factor revealed that the highest values for all traits, except for dry mass, RWC, and essential oil (EO) yield, were observed at the 30–40% field capacity (FC) level, while the lowest values for all traits, except for dry mass, RWC, and EO yield, were recorded at the 90–100% FC level (Table 4). These findings indicate that the imposition of stress at various field capacities resulted in a decrease in trait values, and as the stress intensity increased, the reduction in trait values became more pronounced.

Table 4. Mean comparison of deficit irrigation effect on studied traits

FC (%)	RWC	Antioxidant activity	PROLIN	Soluble sugar	Dry mass	Phenol (mg GAE g^{-1})
90–100	27.61b	32.28d	1.26d	26.11d	23.87a	0.74c
70–80	27.79b	49.48c	1.97c	41.60c	22.43b	1.12b
50–60	30.23a	66.69b	2.76b	50.29b	20.00c	1.22b
30–40	25.53c	73.12a	3.69a	67.80a	15.02d	1.93a

Same letters in each column indicate there is no significant difference between means based on PLSD test.

Table 4 (Continued). Mean comparison of deficit irrigation effect on studied traits

FC (%)	Flavonoid (mg quercetin g^{-1})	Essential oil rate (%)	EO yield g/plant	SOD (µmol min^{-1} mg^{-1} protein)	CAT (µmol min^{-1} mg^{-1} protein)	POD (µmol min^{-1} mg^{-1} protein)	APX (µmol min^{-1} mg^{-1} protein)
90–100	0.23d0	1.15d	0.27b	213.59d	198.53d	159.47d	121.15d
70–80	0.37c	1.22c	0.27b	262.81c	257.06c	206.54c	197.52c
50–60	0.42b	1.58b	0.30a	303.23b	306.59b	232.69b	240.39b
30–40	0.46a	1.70a	0.25c	353.13a	409.09a	270.86a	270.70a

Same letters in each column indicate there is no significant difference between means based on PLSD test

A comparison of melatonin treatments revealed that the application of this hormone led to an increase in the studied traits, except for antioxidant activity and essential oil rate, when compared to the control group. Moreover, increasing the application rate of melatonin resulted in a general increase in most of the studied traits (Table 5). These findings indicate a positive role of melatonin in enhancing the traits and essential oil yield of oregano.

Table 5
Mean comparison of melatonin effect on studied traits

Melatonin (mM)	RWC	Antioxidant activity	PROLIN	Soluble sugar	Dry mass	Phenol (mg GAE g^{-1})
0	24.97b	63.73a	1.90c	48.01b	16.49b	1.08b
100	23.20c	46.80c	2.56b	35.23c	17.13b	1.38a
200	35.21a	55.65b	2.80a	56.11a	27.37a	1.30a
Means in each column with the same letters are not statistically significantly different at $p < 0.05$ based on PLSD test.						

Table 5
(Continued). Mean comparison of melatonin effect on studied traits

Melatonin (mM)	Flavonoid (mg quercetin g^{-1})	Essential oil rate (%)	EO yield g/plant	SOD (µmol min^{-1} mg^{-1} protein)	CAT (µmol min^{-1} mg^{-1} protein)	POD (µmol min^{-1} mg^{-1} protein)	APX (µmol min^{-1} mg^{-1} protein)
0	0.33b	1.56a	0.24b	239.63c	241.25c	186.61b	178.01b
100	0.40a	1.36b	0.23c	288.38b	301.18b	232.15a	212.69a
200	0.38a	1.32b	0.35a	321.56a	336.01a	233.40a	231.62a

Means in each column with the same letters are not statistically significantly different at $p < 0.05$ based on PLSD test.

3.5 Comparison of interaction mean

The comparison of mean interaction effects between stress and hormone treatments indicated that for all traits except antioxidant activity, essential oil rate, and soluble carbohydrates, the application and increased application of the hormone mitigated the effects of stress and increased trait values (Table 6). For instance, the application of 200 mM melatonin concurrently with stress at 70–80% field capacity increased EO yield by 36.86%. The application of 200 mM melatonin concurrently with stress at 90–100% field capacity increased dry mass by 61.01% compared to the no-melatonin treatment and by 42.78% compared to the 100 mM melatonin treatment under stress. Pro content and the enzymatic activities of SOD, CAT, and APX increased by 74.44%, 94.42%, and 54.17%, respectively, with the application of 200 mM melatonin concurrently with stress at 30–40% field capacity compared to the no-melatonin treatment (Table 6).

Table 6
Mean comparison of interaction between stress and melatonin on studied traits

Stress	Melatonin (mM)	RWC	Antioxidant activity	Proline	Soluble sugar	Dry mass	Phenol (mg GAE g^{-1})
90–100% FC	0	21.30de	41.43f	0.88h	32.97fgh	18.40d	0.57g
	100	23.53d	20.70g	1.28g	14.69	20.39c	0.81fg
	200	38.01a	34.71f	1.64f	30.67h	32.83a	0.85ef
70–80% FC	0	21.57de	60.51cd	1.72ef	49.61de	18.43d	1.05def
	100	21.24de	37.60f	2.02de	32.35gh	17.34de	1.11de
	200	40.56a	50.33e	2.16d	42.84def	31.55a	1.21d
50–60% FC	0	28.74c	72.67b	2.06d	59.92bc	15.61ef	1.14d
	100	29.33bc	54.77de	3.05c	41.64efg	16.93de	1.23d
	200	32.62b	72.63b	3.16c	49.31de	27.48b	1.31d
30–40% FC	0	28.25c	80.32a	2.95c	81.95a	13.56fg	1.58c
	100	18.68e	74.13ab	3.87b	52.25cd	13.89f	2.37a
	200	29.66bc	64.92c	4.27a	69.22b	17.64d	1.85b
Means in each column with the same letters are not statistically significantly different at $p < 0.05$ based on PLSD test.							

Table 6
(Continued). Mean comparison of interaction between stress and melatonin on studied traits

Stress	Melatonin (mM)	Flavonoid (mg quercetin g − 1)	Essential oil rate (%)	EO yield g/plant	SOD (µmol min^{-1} mg^{-1} protein)	CAT (µmol min^{-1} mg^{-1} protein)	POD (µ] mol min^{-1} mg^{-1} protein)	APX (µmol min^{-1} mg^{-1} protein)
90–100% FC	0	0.16f	1.16f	0.21de	164.01h	154.44h	108.41f	57.54g
	100	0.26e	1.15f	0.24d	233.21g	207.49g	179.69e	148.13f
	200	0.27e	1.16f	0.38a	243.56fg	233.66fg	190.32de	157.80ef
70–80% FC	0	0.35d	1.17f	0.22de	248.91efg	229.58fg	190.04de	178.00def
	100	0.37d	1.22ef	0.21de	256.43efg	255.98ef	201.06cde	199.12cde
	200	0.40bcd	1.28de	0.41a	283.10def	285.63de	228.52bcd	215.46bcd
50–60% FC	0	0.38cd	1.84b	0.29bc	260.64efg	253.66ef	207.87cde	223.06bcd
	100	0.45b	1.73c	0.29bc	309.73cd	309.32cd	239.94bc	242.81bc
	200	0.44bc	1.19f	0.33b	339.35bc	356.82b	250.26b	255.32ab
30–40% FC	0	0.45b	2.08a	0.28c	284.98de	327.36bc	240.15bc	253.46ab
	100	0.53a	1.34d	0.19e	354.17b	431.97a	307.92a	260.73ab
	200	0.41bcd	1.68c	0.30bc	420.26a	467.95a	264.53b	297.93a

Means in each column with the same letters are not statistically significantly different at $p < 0.05$ based on PLSD test.

The correlation matrix indicated that most traits exhibited high correlations with each other. Essential oil yield showed the highest positive and significant correlation with RWC (r = 0.001) and dry mass (r = 0.780) (Fig. 3). This suggests that an increase in these traits leads to an increase in essential oil yield. A negative and significant correlation was observed between dry mass and RWC with other traits. Biochemical traits (antioxidant activity, proline, soluble sugar, phenol, flavonoid, SOD, CAT, POD, and APX) showed another significant positive correlation. This indicates that an increase in one of these traits leads to an increase in the others.

3.6 Path Analysis

To investigate the precise relationships among traits, path analysis was employed. Path analysis decomposes the relationships between independent and dependent variables into direct and indirect effects, which can significantly aid researchers in accurately interpreting results. When there is a high correlation between traits, this can lead to multicollinearity. In conventional path analysis, multicollinearity can affect the model and lead to the selection of incorrect variables with high errors. Sequential path analysis, which was used in this study, is one method to reduce multicollinearity and simplify the relationships between traits.

The direct effects of the studied traits on dry mass are shown in Table 5. Results indicated that only two models were created in the stepwise regression phase, and the traits RWC and essential oil rate had the greatest impact on determining the essential oil rate (Table 6). The R^2 value for the first model was 0.69 and for the second model was 0.98 (Table 7).

Table 7
Stepwise regression for dry mass Origanum vulgare

Variables entered to model	Step	
	1	2
Intercept	-0.013	15.985
RWC	73.221	79.894
Essential oil rate	-	-12.589
R^2 (%)	60.9	98.9

The trait RWC exhibited the most direct and substantial positive and negative effects, with coefficients of 0.851 and − 0.098, respectively. Similarly, essential oil rate showed the most pronounced indirect and significant positive and negative effects, with coefficients of -0.621 and 0.736, respectively. These findings are detailed in Table 8 and Fig. 4.

Table 8. Direct (diagonal values) and indirect (values outside diagonal) effects of studied traits on dry mass *Origanum vulgare*

Trait	RWC	Essential oil rate	Correlation with dry mass
RWC	0.851	-0.736	0.115
Essential oil rate	-0.621	-0.098	-0.523

4. Discussion

This study found that drought stress led to a reduction in growth and yield parameters for *Origanum vulgare* subsp. vulgare, the sole subspecies examined in this experiment.

Melatonin's foliar application significantly mitigated the adverse impacts of drought stress on *Origanum vulgare* subsp. *vulgare*, enhancing growth, yield, and phytochemical profiles through distinct physiological mechanisms. In this study, melatonin, particularly at 200 mM, upregulated antioxidant enzyme activities —such as SOD, CAT, POD, and APX—which collectively scavenged reactive oxygen species (ROS) like H_2O_2, $^{\cdot}OH$, and O_2^{-}, thereby reducing oxidative damage to cellular components under water deficit conditions (30–40% RAW). This antioxidant enhancement likely stabilized photosynthetic machinery, as evidenced by sustained dry mass under severe drought, contrasting with the reduced biomass observed in untreated plants due to drought-induced photosynthetic inhibition. Additionally, melatonin promoted the accumulation of osmoprotectants such as Pro and soluble carbohydrates, facilitating osmotic adjustment and maintaining cellular turgor, which supported plant survival and essential oil yield under stress. Unlike the untreated controls, where drought stress alone diminished growth parameters, melatonin-treated plants exhibited a dose-dependent resilience, suggesting its role as a signaling molecule that activates stress-responsive pathways. These findings highlight melatonin's direct protective effects, which are more pronounced than the standalone impact of drought stress, offering a mechanistic basis for its potential in improving crop tolerance.

Several studies have utilized dry matter production under stress conditions to evaluate genotype tolerance to stress [33, 34, 35]. In response to increasing drought stress intensity, the leaves of oregano showed a significant decline. The RWC in plants serves as an effective measure for assessing their water status. Under stress, the water consumed by the plant decreases, which may be linked to reduced water-holding capacity in the root environment, diminished water absorption ability, increased resistance in the water flow pathway within the plant, heightened stomatal resistance, and reduced transpiration [36]. Similar reductions in RWC due to stress have also been reported in mint [37], artichoke [38], peppermint [39], marigold [40], and chamomile [41]. In our study, severe drought stress (30–40% field capacity) negatively impacted the vegetative growth of oregano. This detrimental effect likely stems from a water deficit, as drought stress leads to decreased water content, turgor pressure, total water potential, wilting, stomatal closure, and reductions in cell enlargement and vegetative growth [42]. The vegetative growth of plants is dependent on cell division, cell enlargement, and differentiation—all of which are adversely affected by drought stress [43].

One common response to both biotic and abiotic stresses in plants is an increase in the production of osmolytes. To mitigate the negative effects of stress, adapt to new conditions, and protect biological molecules from deformation and destruction, plants enhance their synthesis of certain compounds, such as Pro [44]. Research has shown that fluctuations in Pro levels are linked to a plant's ability to tolerate or adapt to drought stress, making it a useful indicator for selecting drought-tolerant species [45]. When exposed to drought stress, plants accelerate protein degradation, leading to increased concentrations of amino acids and amides, including Pro [46]. Additionally, under stress conditions, plants raise their osmolyte concentrations to maintain water absorption. Among organic osmolytes, Pro is likely the most abundant and widespread compatible solute that accumulates [47]. Pro is stored in the cytoplasm and is believed to play a protective role for intracellular macromolecules during water stress. Pro also serves as an important indicator of a plant's sensitivity to drought stress. The oregano plant likely increases its Pro levels for these reasons. Similar increases in Pro during drought stress have also been reported in thyme (*Thymus vulgaris*). This rise in Pro within plant tissues suggests the activation of an osmoregulatory mechanism that optimizes water and mineral absorption from the root environment. In line with this study, previous research on garden thyme has demonstrated that Pro levels increase under drought stress [48, 49, 36].

One of the biochemical changes that occur in plants under drought stress is the accumulation of reactive oxygen species (ROS). To counteract the oxidative stress induced by such conditions, plant cells activate their antioxidant defense systems [50]. This ability to scavenge ROS represents an adaptive strategy that various plant species employ to cope with oxidative stress [51]. In this study, the total antioxidant activity of oregano plants was observed to increase with the severity of drought, suggesting a defense mechanism to protect them during stressful conditions. Typically, when plants face biotic and abiotic environmental stresses, ROS levels quickly rise as an initial line of defense [52]. While these oxidative ROS play a role in maintaining proper cellular homeostasis, excessive production can lead to cell damage and death [53]. To manage such situations, plants enhance their antioxidant activity by producing antioxidant enzymes, which create an efficient system for maintaining ROS homeostasis in various parts of the cell, both under normal and stressful conditions [54]. In this study, the increase in total antioxidant activity contributed to maintaining cellular homeostasis in oregano plants. In a related experiment on the medicinal plant Portulaca oleracea, it was also found that antioxidant activity increased under drought stress [55].

Under high irrigation levels, the application of hormones effectively mitigated the effects of drought on oregano plants. Specifically, the highest activity of antioxidant enzymes was observed with the application of 200 mM melatonin under conditions where the field capacity was reduced to 40%. This finding suggests that oregano plants can maintain cellular homeostasis by altering the utilization mechanisms of melatonin when facing drought stress at 60% field capacity, possibly through the production of other osmolytes. During drought stress, the level of ROS increases in the cytoplasm of plant cells, potentially damaging intracellular organelles such as chloroplasts, mitochondria, and peroxisomes. However, the application of melatonin under these conditions enhances the production of both enzymatic and non-enzymatic antioxidants, thereby protecting the plant from the adverse effects of drought [56]. Additionally, under drought conditions, anti-stress hormones serve as chemical messengers. By activating secondary messengers like ROS, they help establish a defensive mechanism against stress [57]. Correspondingly, research on sesame plants has shown that melatonin application during drought stress increases total antioxidant activity [56].

The data on secondary metabolites, particularly the essential oil components of *Origanum vulgare*, revealed significant variations in response to drought stress and melatonin treatment, as detailed in Table 2. Notably, compounds such as linalool, terpinolene, caryophyllene, and farnesene increased significantly under severe drought (30–40% RAW) with 200 mM melatonin application, suggesting a stress-induced biosynthetic shift modulated by melatonin. This increase aligns with the plant's adaptive strategy to enhance volatile compound production under water deficit, potentially serving as a protective mechanism against oxidative damage or as signaling molecules. In contrast, other compounds like α-thujene and limonene showed less consistent changes across

treatments, indicating differential regulation within the terpenoid pathway. These findings align with previous reports on oregano, where drought stress elevates phenolic monoterpenes like carvacrol [6], and suggest that melatonin may selectively upregulate specific secondary metabolite pathways, offering a promising avenue for improving essential oil quality under adverse conditions.

Plant resistance to various environmental stresses is often linked to the activity levels of enzymes responsible for scavenging free oxygen radicals. The antioxidant response to water deficiency varies depending on the severity of the stress and the plant species [16]. Generally, resistant plant species exhibit a more efficient protective capacity against oxidative stress induced by water stress; this capacity can be enhanced by increasing the activity of antioxidant enzymes [58, 15]. When plants undergo water deficiency, activating their defense systems to combat damage from oxidative stress becomes essential. Consequently, the activities of SOD, POD, APX, and CAT increase in response to various environmental stresses [59]. In a study involving lavender plants subjected to drought stress, the plants demonstrated a highly efficient system for maintaining reactive oxygen species (ROS) homeostasis by elevating the induction of antioxidant enzymes such as SOD, POD, APX, and CAT. This response helps protect them against drought-induced damage [60]. The induction of antioxidant enzyme activity is a natural adaptation strategy plants use to cope with oxidative stress [54]. Among these antioxidant enzymes, SOD is recognized as the most crucial enzyme for responding to free radicals (ROS) by converting them into hydrogen peroxide (H_2O_2) and promoting the activity of other enzymes [61]. Superoxide dismutase regulates the concentrations of hydrogen peroxide and superoxide, making it a critical component of the antioxidant defense system [62]. The increase in SOD activity, along with the mechanisms to inhibit H_2O_2, such as those mediated by CAT and POD, constitutes an anti-drought mechanism [63]. APX plays a role in detoxifying hydrogen peroxide generated by the activity of SOD. The study also found significant differences in the activity of antioxidant enzymes in the aerial parts of oregano when subjected to drought stress and treatment with melatonin. Experimental evidence suggests that APX is more unstable than SOD under oxidative stress conditions [64]. Overall, the comparison of antioxidant enzyme activity under drought stress revealed a significant increase in the activities of SOD, CAT, POD, and APX at all levels of applied drought stress. Additionally, research conducted on the plant *Echinacea purpurea* indicated that drought stress (following 60% depletion of soil moisture) elevated the activity of antioxidant enzymes such as SOD, CAT, POD, and APX [65]. Similarly, studies have shown that drought conditions enhance the activities of SOD and CAT enzymes at varying stress levels in maize [66], soybean [67], rice [61], and fennel [59]. Figure 5 illustrates the potential mechanism for melatonin-induced mitigation of drought effects in *O. vulgare*, based on the results of this study and related literature.

Correlation and regression analyses are commonly employed to identify relationships between variables and play a crucial role in agricultural research [68]. In their study, Salehi Sardoei and colleagues (2023) reported significant positive correlations between growth and yield traits in citrus cultivars [68]. These correlations can help plant breeders indirectly select important stress-related traits by measuring other traits that are easier to assess [69]. While correlation coefficients for physiological traits are useful for identifying traits linked to stress, they do not accurately describe the relationships between these traits. Therefore, it is essential to determine the direct and indirect effects of these traits in plant breeding programs [70]. Stepwise regression can be employed to identify the most significant variables among numerous measured traits, thus clarifying their contributions to explaining tolerance [69]. This method can eliminate ineffective traits from the model and focus on those that account for a significant portion of the variation [71]. In the present study, several traits significantly correlated with dry mass, prompting further investigation using additional statistical methods to identify the traits influencing yield. To gain deeper insights into the relationships among the studied traits and their effects on dry mass, path analysis was used. Stepwise regression determined the most influential traits related to dry mass and excluded insignificant traits prior to conducting path analysis. The R^2 values for the first and second models were 0.69 and 0.98, respectively. The results from the stepwise regression indicated that the first two variables entered into the model, RWC and essential oil rate, contributed most significantly to explaining dry mass. To interpret the outcomes of simple correlation and stepwise regression more effectively, the variables in the model underwent path analysis. The highest positive effect was attributed to RWC (0.851), which has a positive correlation with dry mass (0.115). This suggests that RWC directly influences dry mass, with negligible indirect effects from other traits. Consequently, RWC can be utilized as a selection criterion in oregano breeding programs.

5. Conclusion

The current study demonstrates that the application of melatonin can help mitigate the negative effects of water deficit stress on oregano plants. This approach may reduce the need for chemical fertilizers and promote sustainable agricultural practices. The findings revealed that as water deficit stress intensified, the dry weight of the oregano plants decreased. However, levels of proline, soluble carbohydrates, antioxidant activity, and the activities of certain enzymes increased, indicating the plants' adaptive response to the stress. When melatonin was applied at a concentration of 200 mM, the plants exhibited improved resistance to water deficit stress, as evidenced by enhanced antioxidant activity and increased activity of antioxidant enzymes. Overall, the study highlights the significance of melatonin application in inducing various mechanisms of drought tolerance in oregano plants.

The findings of this study not only underscore the efficacy of melatonin as a drought stress mitigator in oregano but also contribute to the broader understanding of its role as a versatile phytohormone in stress physiology. By demonstrating how melatonin modulates antioxidant defenses and osmoprotectant accumulation under varying drought intensities, this research advances the potential for its application in other medicinal and aromatic plants facing water-limited conditions. Furthermore, these insights pave the way for future investigations into the molecular mechanisms underlying melatonin's protective effects, potentially leading to the development of targeted, eco-friendly strategies for improving crop resilience in arid and semi-arid agricultural systems.

Declarations

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Authors' Contributions

Hossein Gorgini Shabankareh (H.G.S.) designed and conducted the experiment, collected data, and performed initial analyses. Mohammad Reza Asgharipour (M.R.A.) and Ali Salehi Sardoei (A.S.S.) contributed to the experimental design, data interpretation, and wrote and revised the manuscript. Fatemeh Gorgini Shabankareh (F.G.S.) provided statistical analysis and technical support for data processing. All authors reviewed and approved the final manuscript.

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Data availability

The data produced or analyzed during the study are accessible from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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Figures

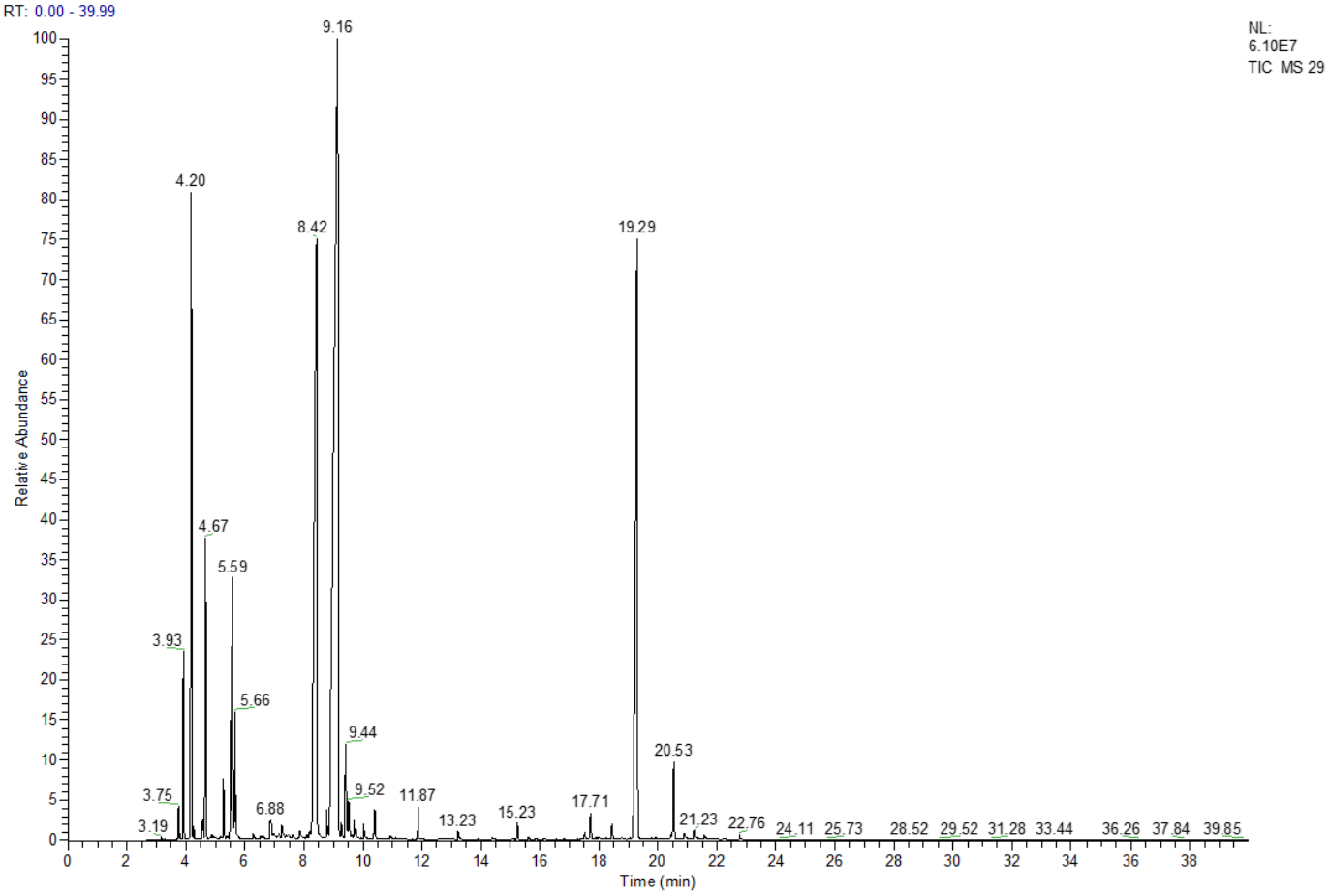

Figure 1

Chromatogram showing the secondary compounds of oregano under 60% RAW and 200 mM hormone treatment

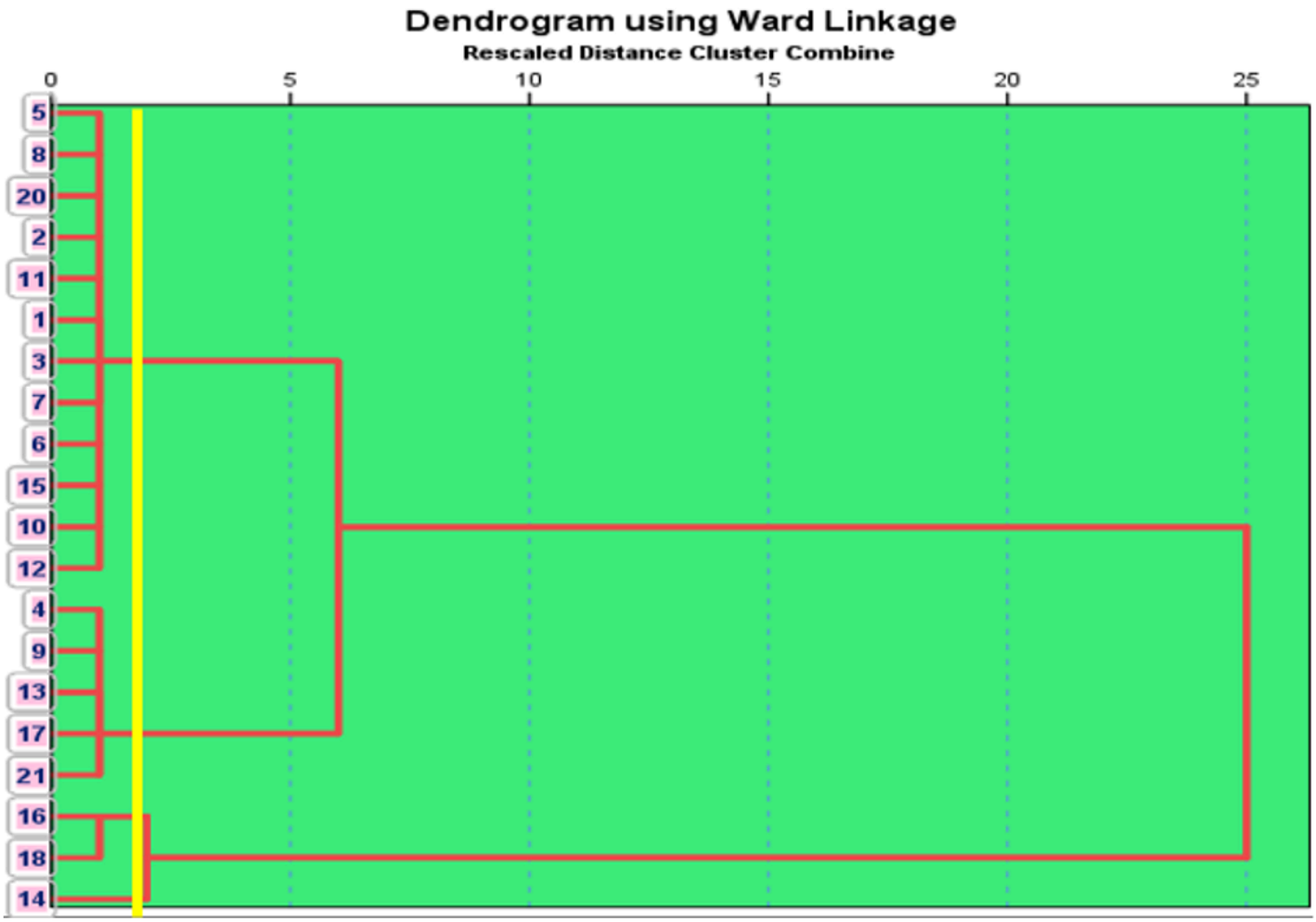

Figure 2

Dendrogram illustrating the genetic relationships based on identified compounds in oregano plants. Attributes were aligned using the CLUSTAL W method and grouped using the WARD method.

Y axis are respectively: 1- α-Thujene; 2- α-pinene; 3- Camphene; … 21- Caryophyllene oxide.

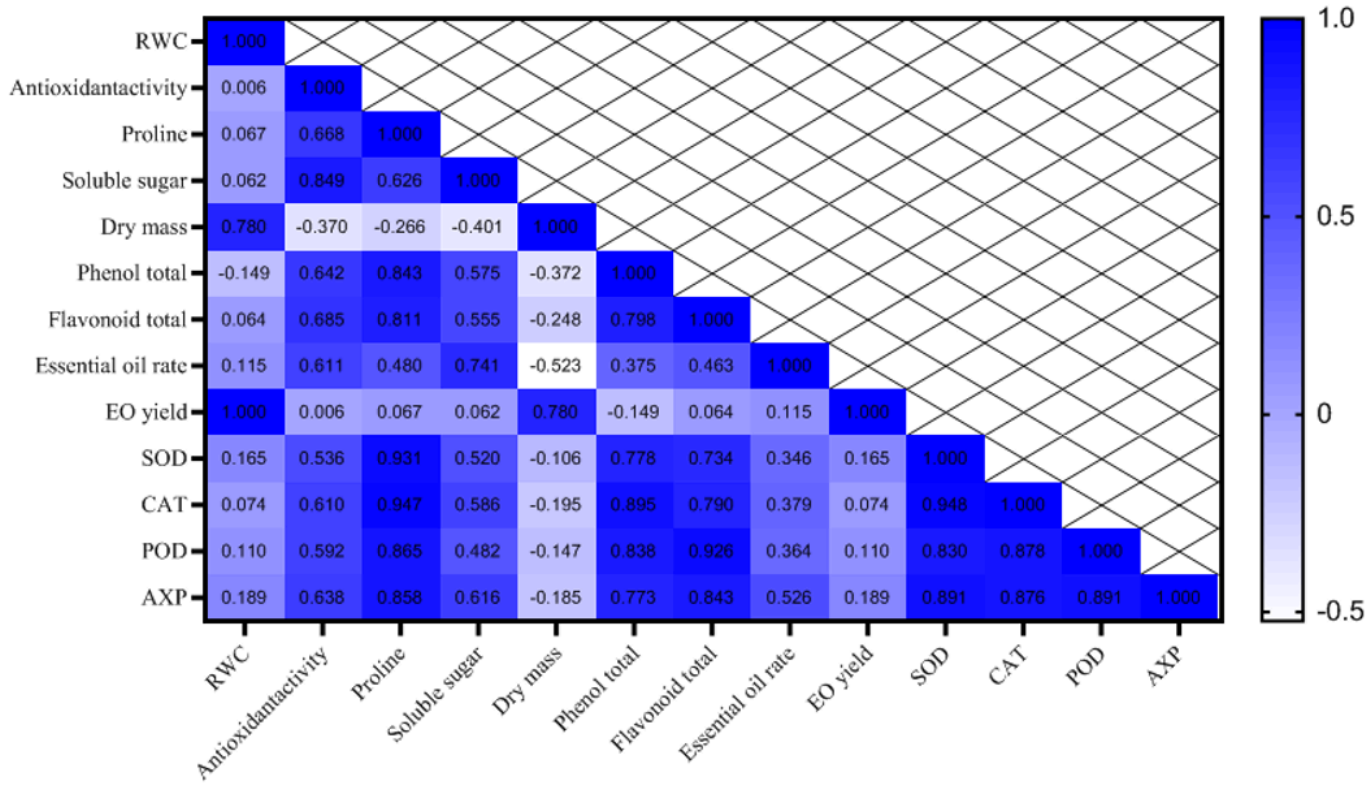

Figure 3

Heat map of mutual relations of variables in correlation coefficient for studied traits

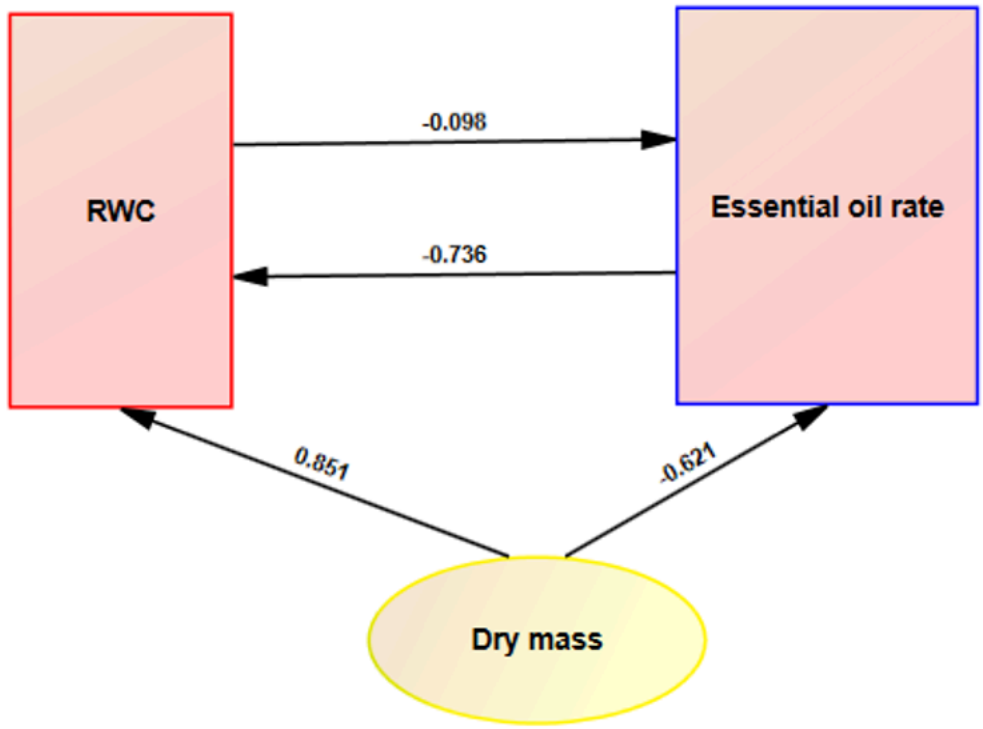

Figure 4

A sequential model illustrating the relationships between dry mass, relative water content (RWC), and essential oil rate in oregano.

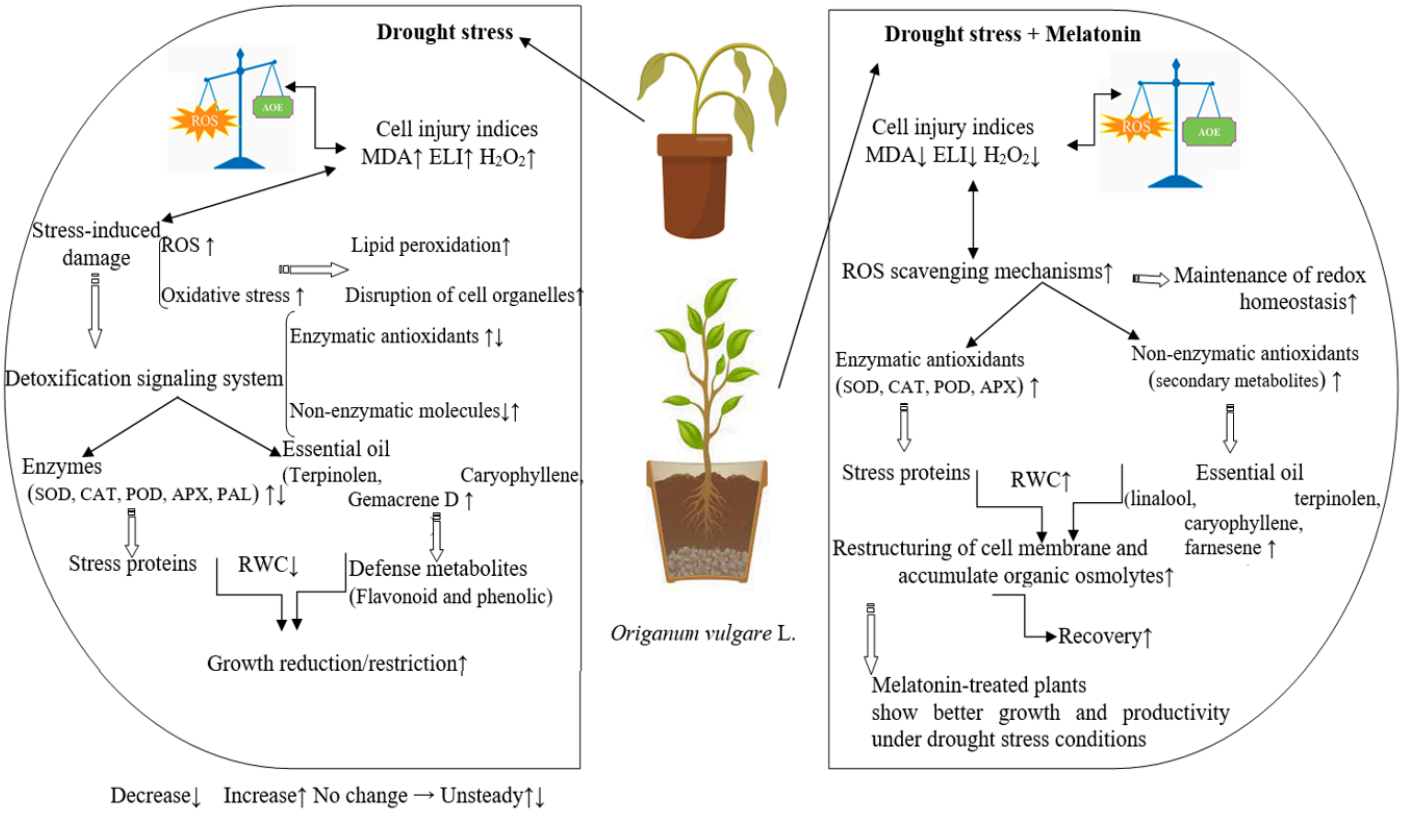

Figure 5

The proposed mechanism outlines how melatonin helps reduce the negative effects of drought in *Origanum vulgare*L. sp. vulgare. Drought stress leads to an overproduction of reactive oxygen species (ROS) such as H_2O_2, $^{\cdot}OH$, and O_2^{-}, as well as the inhibition of chlorophyll biosynthesis, resulting in decreased growth and metabolism. Exogenous melatonin can mitigate drought stress through several mechanisms. Firstly, it can upregulate both enzymatic (e.g., SOD, POX, CAT, APX, GR) and non-enzymatic (e.g., phenolics and flavonoids) antioxidant molecules, which help in scavenging ROS and preventing oxidative damage in plant cells. Secondly, melatonin can aid in the accumulation of osmoprotectants, thereby maintaining organic compounds. Additionally, applied exogenous melatonin can act as an antioxidant agent, combatting ROS and lipid peroxidation, and ultimately ensuring the survival of plants under water deficit stress.