horticulturae

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Exogenous β-Aminobutyric Acid (BABA) Improves the Growth, Essential Oil Content, and Composition of Grapefruit Mint (*Mentha suaveolens* × *piperita*) under Water Deficit Stress Conditions

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Abstract: Grapefruit mint, *Mentha suaveolens* × *M. piperita*, belonging to the Lamiaceae family, is an important medicinal plant with applications in the cosmetic, pharmaceutical, food, and nutraceutical industries. Environmental factors such as cold, salinity, and water deficit significantly impact the quantity and quality of the active compounds of medicinal plants. To examine the effects of drought stress and β-aminobutyric acid (BABA) as an elicitor on the biochemical characteristics and essential oil (EO) profile of grapefruit mint, a factorial experiment was conducted in a completely randomized design (CRD) with two factor and three replications under greenhouse conditions. The first factor included field moisture capacity (FC) as the control (100% FC), mild (75% FC), moderate (55% FC), and severe water deficit stress (35% FC), while the second factor consisted of 0 (control plants without BABA), 0.8, 1.6, and 2.4 mM of BABA foliar application. Water stress and BABA application significantly affected the EO content and composition of grapefruit mint. The highest content of EO was observed in mild drought stress and BABA spraying at 1.6 to 2.4 mM, which increased by about 140% compared with the control condition. The EO components were identified using GC-FID and GC-MS analysis. Linalool (33.7–47.3%) and linalool acetate (31.2–52%) were the most abundant compounds. The highest content of linalool acetate was observed in severe drought stress (35% FC) with foliar application of BABA (1.6 mM), which increased by 33.86% compared with the control condition. However, the highest content of linalool was observed under normal irrigation with foliar application of 0.8 to 1.6 mM BABA. Based on the results, severe drought stress reduced the total chlorophyll and carotenoids by 81.76 and 64.6% compared with the control condition, respectively. Water stress and the foliar application of BABA significantly affected the activity of antioxidant enzymes (ascorbate peroxidase, APX; guaiacol peroxides, GPX; and superoxide dismutase, SOD). The application of 1.6 mM BABA significantly increased the activity of antioxidant enzymes under water stress conditions. Finally, our results showed that the application of BABA (mainly at 1.6 mM) can improve the grapefruit mint yield and EO profile under water stress conditions.

Keywords: grapefruit mint; essential oil; dry weight yield; linalool; linalyl acetate; β-aminobutyric acid

Citation: Akbarzadeh, S.; Morshedloo, M.R.; Behtash, F.; Mumivand, H.; Maggi, F. Exogenous β-Aminobutyric Acid (BABA) Improves the Growth, Essential Oil Content, and Composition of Grapefruit Mint (*Mentha suaveolens* × *piperita*) under Water Deficit Stress Conditions. *Horticulturae* **2023**, *9*, 354. https://doi.org/10.3390/horticulturae9030354

Academic Editors: Jelena Popović-Djordjević, Luiz Fernando Cappa de Oliveira, Haroon Khan and Sina Siavash Moghaddam

Received: 19 February 2023
Revised: 2 March 2023
Accepted: 5 March 2023
Published: 7 March 2023

1. Introduction

The genus *Mentha* (Lamiaceae) contains about 30 herbaceous, fast-growing species that are extensively distributed and cultivated in most areas of the world [1,2]. Grapefruit mint (*Mentha suaveolens* × *M. piperita*) is a perennial herb with large oval crinkled leaves and sporadic glandular hairs in the upper surface of the leaves, releasing a strong citrusy

scent [3]. The plant is a sterile hybrid of *M. suaveolens* Ehrh. and *M* × *piperita* L. [4,5]. Grapefruit mint is widely used as a flavoring agent and spice in food production and beverages [4,6]. It has been reported that the aqueous extract of grapefruit mint has anticytopathogenic and HIV-1 reverse-transcriptase-suppressing activity [7]. Its leaves are a rich source of essential oil (EO) with potential use in food, oral hygiene products, syrups, and ice cream production [8,9]. The major compounds of grapefruit mint EOs are linalool, linalool acetate, and linalyl anthranilate [4,10–12]. In the mint family, grapefruit mint is one of the few species with linalool as the predominant EO component [10].

EOs have an important role in the biological activities of plants, and their production and accumulation are highly dependent on environmental and genetical factors [13–15]. Therefore, applying stress can be an effective way to improve the production of secondary metabolites in many species of medicinal plants [16,17]. The agricultural industry has always been threatened by various environmental stresses, which are recognized as a major cause of crop damage around the world [18]. Nowadays, due to global climate change and a decrease in rainfall, Middle Eastern countries are facing a significant reduction in water resources leading to numerous biotic and abiotic stresses in plants [19]. Drought stress is considered as one of the most important abiotic stresses because it changes the structure of the cellular components, reduces photosynthesis, increases the production of reactive oxygen species (ROS), and disrupts the functioning and plant growth [20,21]. Interestingly, previous studies demonstrated that medicinal plants grown in semi-arid climates usually contain higher concentrations of active compounds than similar species in temperate climates, referring to a defense mechanism against stressful conditions [22]. In a study by Alhaithloul et al. [23], the growth rate and biomass of *M* × *piperita* L. and *Catharanthus roseus* (L.) G.Don were significantly reduced under drought stress, and the plants increased the production and accumulation of their secondary metabolites under this condition [23].

Elicitors are a class of chemical compounds with a low molecular weight that improve the plants immune response by stimulating secondary metabolites production and stress responses and play an important role in plant adaptation to stress conditions [24,25]. These compounds can be applied in all plant life cycles and affect the plant's reactions and growth [26]. Elicitors usually have a low cost, can be easily applied, and can considerably improve the plant's tolerance to stress [27]. It is notable that the induction or enhancement of the biosynthesis of secondary metabolites can be accomplished through small amounts of elicitors [28]. Improving the production of secondary metabolites using elicitor compounds under stress circumstances guarantees the survival, stability, and competitiveness of medicinal plants, which can have significant economic benefits for farmers [29].

β-Aminobutyric acid (BABA) is a non-protein amino acid elicitor which induces resistance in plants [30]. Interestingly, it has been reported that this induced resistance can be transferred to the next generations [31]. A previous study reported that the exogenous application of BABA can significantly increase plant tolerance to biotic and abiotic stresses such as salinity and drought [32]. BABA is adsorbed systematically into the plant and induces different physiological and biochemical changes in plant metabolism [31]. In an experiment performed to measure the amount of BABA in plant tissues using liquid chromatography (LC-MS/MS), the results showed that BABA was naturally present in *Arabidopsis thaliana* (L.) Heynh., *Triticum aestivum* L., *Brassica rapa* L., *Physcomitrella patens* (Hedw.) Bruch and Schimp., and *Zea mays* L. [33]. In a study, the application of BABA in cherry fruit reduced the level of polygalacturonase and pectin methyl esterase activity and increased the amount of cell wall polysaccharides by reducing membrane permeability and malondialdehyde (MDA) content; thus it integrates the structure of the cell epidermis and leads to a delay in the aging process [34]. It was reported that the application of BABA in *Arabidopsis* plants under salinity stress leads to an increase in abscissic acid accumulation and the expression of stress-regulating genes, as a result of the plant's resistance to pathogens enhancement [35]. On the other hand, the exogenous application of BABA can stimulate the production of osmolytes, improve the activity of antioxidant

enzymes, and enhance their transcripts, which results in an increase in the osmotic balance in the plant subjected to drought stress [36].

Due to the high medicinal value and economical importance of grapefruit mint, the present study was aimed at investigating the effect of different concentrations of BABA on drug yield, EO content and composition, linalool and linalool acetate content, and the physiological parameters of the plant under water deficit stress. As previously mentioned, there are some reports which refer to the elicitor effects of BABA in plant species, but there is no comprehensive work to show the effect of BABA on the essential oil production of medicinal plants under water deficit stress conditions. Water deficit stress affects the quality and quantity of the plant; thus, the study was aimed at investigating the theoretically optimized concentration of BABA for the high-throughput production of grapefruit mint and reducing the negative effects of drought stress.

2. Materials and Methods

2.1. Experimental Design

Here, a factorial experiment based on a completely randomized design (CRD) with two factors (i.e., the interaction effects of three levels of water deficit stress and three levels of BABA foliar spray), and three replications was performed. The study was conducted in the glass greenhouse of the University of Maragheh, Iran (37°30′ N, 46°12′ E, altitude 1477.7 m a.s.l). The first factor included three water deficit stress levels including: field moisture capacity (FC) as the control (100% FC), mild (75% FC), moderate (55% FC), and severe stress (35% FC), while the second factor encompassed the four levels of foliar spraying of BABA at 0, 0.8, 1.6 and 2.4 mM.

2.2. Appling Water Deficit Stress and BABA Spraying

Grapefruit mint rhizomes were propagated in 48-cell transplant trays containing cocopeat: perlite mixture (2:1 ratio, v:v). Then, the transplants with eight pair-leaves (10–12 cm) were transferred into 5 L pots containing a mixture of sieved agricultural soil, silt, manure, and perlite (50:25:15:10, v:v). To warrant the normal plants' growth, they were subjected to a temperature of 19–28°C, a photoperiod of 16 h, and relative humidity of 50–80%. Before starting the water deficit stress, the plants were irrigated regularly. After that, the transplants were established (twenty days after planting) and the grapefruit mint clones were exposed to four levels of water deficit stress. To apply the water deficit stress treatment, the pots were weighed daily, and the procedure outlined by Morshedloo et al. [37] was used for adding replacement water. Briefly, the percentage of soil water content was measured 24 h after watering. Then, 100 g of soil sample collected from the pot under each water stress condition was oven dried and reweighed. Any additional water determined as necessary to bring the soil to the designated stress moisture content was added to all the pots within that stress level.

On the other hand, the foliar application of BABA started three days before the water deficit stress treatments (to activate the plant's defense mechanisms) and, as mentioned before, four concentrations (control, 0.8, 1.6, and 2.4 mM) were applied. The control plants were sprayed using distilled water. In order to prevent nutritional deficiency, the grapefruit mints were irrigated four times during the growth stages with Hogland solution [38]. Subsequent BABA foliar sprays were conducted 20 days apart from each other. Finally, before harvesting, the plants with fully expanded leaves were kept at −80 °C for biochemical analyses.

2.3. Growth Parameters and Photosynthetic Pigments Assay

To obtain the fresh and dry weight, the treated plants were harvested at the full flowering stage by cutting the stem 5 cm above the soil. After measuring the fresh weight, the plants were completely dried in the shade for 7 days and weighed to determine the dry weight. The content of chlorophyll a, chlorophyll b, and carotenoids were measured based on the Arnon [39] method. For this purpose, 0.2 g of fresh frizzed leaf sample were extracted in 10 mL 80% acetone solution, then centrifuged for 15 min at 10,000 rpm.

Subsequently, the absorbance of the supernatant was measured at 663, 645, and 470 nm, and the concentrations of photosynthetic pigments were calculated using the equations described by Arnon [39].

2.4. Total Soluble Protein Content (TSPC)

In order to measure the total protein content of grapefruit mint under the mentioned treatments, 0.2 g of frizzed leaves were extracted with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.5; 2% (*w*/*v*) PVP; 1 mM EDTA). The mixture was centrifuged at 16,000 rpm for 15 min at 4 °C. Subsequently, the supernatants were applied for a TSPC assay. The TSPC was investigated according to the method outlined by Bradford [40]. BSA was used as a standard and the absorbance was recorded at 595 nm.

2.5. Enzymatic Antioxidants Activity

Ascorbate peroxidase (APX) activity was determined according to the procedures outlined by Miyake and Asada [41] based on ascorbic acid (AA) oxidation at 290 nm. The reaction composition included 0.5 mM ascorbate in a potassium phosphate buffer (50 mM). The data were reported as μmol asc. min^{-1} mg^{-1} protein.

Guaiacol peroxidase activity (GPX) activity was assessed by decreasing H_2O_2 with the oxidation of guaiacol based on the method described by Morshedloo et al. [13]. The reaction mixture comprised 480 μL of guaiacol (20 mM) in a potassium phosphate buffer (50 mM) pH:7, and H_2O_2 (3%). The increase in absorbance was recorded at 470 nm over 60 s and the GPX activity was reported as μmol H_2O_2 min^{-1} mg^{-1} protein.

Superoxide dismutase activity (SOD) activity was measured based on the Beauchamp and Fridovich [42] method with minor modifications. To assay the SOD activity, 50 μL from the enzyme extract were added to 2.95 mL of reaction mixture including 1.5 mM sodium carbonate, 3 mM EDTA, 0.2 M methionine, 0.1 M K-phosphate buffer, and 2.2 mM nitroblue tetrazolium (NBT). Lastly, the reaction was started by adding 100 μL of riboflavin (60 μM). The reaction mixture was placed at 25°C for 15 min under a fluorescent light. The absorbance was measured at 560 nm. The SOD activity was reported as units min^{-1} mg^{-1} protein.

2.6. Total Proline Content

Ninhydrin was used to determine the proline content of the treated plants. For this purpose, 0.5 g of fresh frizzed leaves were mixed with 10 mL of 3% sulphosalicylic acid; then, the samples were centrifuged for 20 min at 12,000 g. Subsequently, 2 mL of glacial acetic acid and 2 mL of ninhydrin were added to the supernatant obtained from the centrifugation of the samples and heated in a hot water bath for 60 min. The mixture was instantly cooled on ice for 5 min, then extracted with 4 mL of toluene. The absorbance was recorded at 520 nm [43].

2.7. Malondialdehyde (MDA) Content

The MDA content was measured based on the Heath and Packer [44] method, where 0.5 g of fresh frizzed leaf samples was homogenized with 1.5 mL of trichloroacetic acid 1%, then centrifuged at 12,000× *g* for 10 min. After that, 1 mL thiobarbituric acid 0.1% was added to 500 μL of the supernatant obtained from the centrifuge. The resulting composition was heated in a hot water bath at 95°C for 30 min, then immediately cooled on ice for 15 min. Eventually, the absorbance was read at 532 and 600 nm. The content of MDA was expressed as nmol g^{-1} fresh weight (FW).

2.8. Essential Oil (EO) Extraction

The dried flowering aerial parts (20 g) of all experimental pots (n = 3) were exposed to hydrodistillation using a Clevenger apparatus for 3 h according to the method introduced in European pharmacopeia. Then, to obtain pure EOs, the redundant water of the samples was removed using anhydrous sodium sulfate. Finally, the EO content was obtained as the ratio of EO volume to dry weight (*v*/*w*) of plant samples [45].

2.9. GC-FID and GC-MS Analysis

For the gas chromatography–mass spectrometry (GC–MS) analysis, an Agilent 7990B/5977A series was used. For the separation of EO components, the instrument was equipped with an HP-5MS capillary column. Gradient temperature was used for ideal separation as follows: the injector temperature sets at 240 °C; transfer line temperatures sets at 250 °C; and the oven temperature was set at 60 °C for 5 min, then raised to 230 °C with rate of 3 °C per min. The carrier gas in this experiment was helium (1 mL min^{-1}). The injector was in split mode with a split ratio of 1:30. The mass detector scanned through the range of 40–450 m/z. To calculate the retention indexes of the components, a homologous series of hydrocarbons (C_8-C_{40}, Supelco, Bellefonte, USA) was used. For each compound, the calculated retention index (RI) was compared with those reported in the reference literature [46], and the interpretation of mass data within the WILEY275 and NIST 05 libraries. For some of the main components, the identification process was validated through a peak assignment by co-injecting available authentic standards [4]. A GC (Agilent 7990B) instrument coupled with an FID was used for the semi-quantitative analysis of the EO compounds. The column used in the GC instrument was VF-5MS. The separation process was the same as described above for the GC-MS analysis. For quantification, the internal peak areas of each EO component were integrated [47].

2.10. Statistical Analysis

All analyses were conducted on a factorial experiment based on a completely randomized design (CRD) with two factors and three replications performed. The analysis of variance (ANOVA) was performed using the MSTAT-C software [Michigan State University, USA] followed by a least significant difference test (LSD; p value < 0.05). The mean values are reported with the standard errors (n = 3). Pearson's correlation among the treats was assessed using IBM SPSS Statistics 23.0 (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Growth Characteristics

The results demonstrated that water deficit stress and BABA had significant effects on the growth parameters. So, the highest amount of fresh weight (111.6 g pot^{-1}) was observed in the control condition with a foliar application of BABA at 2.4 mM. On the other hand, the lowest amount of fresh weight (30 g pot^{-1}) was observed in the severe drought stress conditions without a BABA application, which was reduced by about 70%, compared with the control plants (Table 1). However, under severe drought stress, the dry weight decreased by 63.07% compared with the control plants. BABA foliar application affected the dry weight (DW). Thus, with a foliar application of BABA at 2.4 mM, the DW increased by 23.05% compared with a distilled water application (Table 2). Drought stress leads to changes in the biochemical and morphological characteristics of plants, and these changes usually improve the plants' tolerance to the created conditions [19]. In the present study, water stress decreased the amount of fresh and dry weight, while BABA foliar application adjusted the growth indicators of grapefruit mint under water stress and could improve plant growth under the stress condition. Water stress might directly influence photosynthesis by affecting photochemical processes in the leaf, and indirectly by closing the stomata and reducing the leaf area, so reducing the plant weight [48]. A reduction in plant growth would increase the possibility of its survival under water deficit stress and is presumed to be a mechanism for drought tolerance [48]. The reduction of the leaf area in plants is known as the first mechanism to deal with drought stress [49]. Interestingly, Jakab et al. [35] reported that the foliar application of BABA in the *Arabidopsis* plants increases the drought tolerance by increasing the accumulation of abscisic acid and reducing the size of the stomata [35], and subsequently improves plant growth. However, BABA application in high concentrations limits the growth of the lateral organs in *Arabidopsis* by decreasing the cell division in the meristem tissues [50]. Consistent with our findings, in the studies of Mohammadi et al. [51] on *Thymus vulgaris* L. and Baghbani-Arani et al. [52]

on *Trigonella foenum-graecum* L., the authors showed that water stress limits the growth and development of plants and reduces their performance.

Table 1. Effects of the foliar application of BABA on the growth and physiological parameters in grapefruit mint plants under different water stress.

Water Stress	BABA (mM)	Fresh Weight (g Pot^{-1})	Chlorophyll *a* (mg g^{-1} Fresh Weight)	Chlorophyll *b* (mg g^{-1} Fresh Weight)	Total Chlorophyll (mg g^{-1} Fresh Weight)	Carotenoid (mg g^{-1} Fresh Weight)	MDA (nmol g^{-1} Fresh Weight)	APX (µmol asc. Min^{-1} mg^{-1} Protein)	SOD (Units min^{-1} mg^{-1} Protein)
100% FC (Control)	0	100 ± 0.58 c *	5.03 ± 0.01 a	1.88 ± 0.01 abc	6.91 ± 0.01 abc	1.79 ± 0.03 d	0.33 ± 0.01 hij	0.2 ± 0.02 j	15.13 ± 1.17 h
	0.8	100 ± 0.00 c	5.06 ± 0.01 a	1.91 ± 0.01 ab	6.91 ± 0.01 ab	2.06 ± 0.11 c	0.31 ± 0.04 ij	0.25 ± 0.01 ij	21.49 ± 1.44 g
	1.6	103.33 ± 0.88 b	5.09 ± 0.02 a	1.94 ± 0.01 a	7.04 ± 0.02 ab	2.48 ± 0.05 b	0.31 ± 0.01 ij	0.23 ± 0.01 ij	23.15 ± 1.45 g
	2.4	111.66 ± 0.88 a	5.23 ± 0.05 a	1.95 ± 0.00 a	7.18 ± 0.05 a	2.64 ± 0.06 a	0.26 ± 0.01 j	0.31 ± 0.05 ij	26.22 ± 1.8 g
75% FC (Mild)	0	80 ± 0.00 g	4.96 ± 0.00 a	1.65 ± 0.01 de	6.62 ± 0.01 abcd	1.62 ± 0.01 efg	0.65 ± 0.01 d	0.51 ± 0.05 hi	26.82 ± 1.53 g
	0.8	90 ± 0.58 f	4.97 ± 0.00 a	1.72 ± 0.03 d	6.7 ± 0.03 abcd	1.67 ± 0.02 def	0.43 ± 0.05 gh	0.75 ± 0.08 gh	40.65 ± 1.67 f
	1.6	95 ± 0.58 e	4.99 ± 0.01 a	1.81 ± 0.00 c	6.8 ± 0.01 abc	1.72 ± 0.01 de	0.38 ± 0.05 hi	0.83 ± 0.07 g	51.62 ± 1.23 de
	2.4	96.66 ± 0.33 d	5.02 ± 0.00 a	1.85 ± 0.01 bc	6.86 ± 0.01 abc	1.77 ± 0.00 d	0.36 ± 0.02 hi	1.14 ± 0.03 f	38.88 ± 1.21 f
55% FC (Moderate)	0	56.66 ± 0.33 k	4.9 ± 0.01 a	1.28 ± 0.05 h	6.18 ± 0.06 d	1.49 ± 0.01 hi	0.87 ± 0.02 c	1.23 ± 0.03 ef	40.79 ± 1.68 f
	0.8	63.33 ± 0.33 j	4.93 ± 0.00 a	1.46 ± 0.02 g	6.38 ± 0.02 cd	1.51 ± 0.01 ghi	0.6 ± 0.04 de	1.39 ± 0.04 def	53.43 ± 1.63 d
	1.6	66.66 ± 0.33 i	4.93 ± 0.00 a	1.54 ± 0.02 fg	6.47 ± 0.02 bcd	1.54 ± 0.00 ghi	0.34 ± 0.05 hij	1.59 ± 0.02 cd	60.3 ± 1.57 c
	2.4	75 ± 0.58 h	4.95 ± 0.00 a	1.6 ± 0.02 ef	6.56 ± 0.02 bcd	1.58 ± 0.01 fgh	0.49 ± 0.05 fg	1.63 ± 0.22 cd	51.39 ± 0.28 de
35% FC (Severe)	0	30 ± 0.58 o	1.12 ± 0.27 c	0.13 ± 0.01 l	1.26 ± 0.29 g	0.63 ± 0.06 k	1.55 ± 0.04 a	1.48 ± 0.1 de	47.43 ± 3.9 e
	0.8	39 ± 1 n	3.96 ± 0.7 b	0.22 ± 0.06 k	4.18 ± 0.74 f	1.22 ± 0.09 j	1.06 ± 0.03 b	1.8 ± 0.04 bc	64.49 ± 2.37 bc
	1.6	41.66 ± 0.33 m	4.83 ± 0.02 a	0.44 ± 0.03 j	5.27 ± 0.05 e	1.44 ± 0.00 i	0.69 ± 0.03 d	2.28 ± 0.29 a	78.33 ± 3.49 a
	2.4	46.66 ± 0.33 l	4.87 ± 0.00 a	0.62 ± 0.07 i	5.49 ± 0.07 e	1.45 ± 0.00 i	0.54 ± 0.01 ef	1.98 ± 0.03 b	66.19 ± 2.99 b
LSD		1.59	0.54	0.08	0.57	0.12	0.09	0.29	5.88

* In each row, means with the same letter do not have a significant difference ($p < 0.05$) with each other according to the LSD mean comparison test.

Table 2. Simple effects of foliar application of BABA and water stress on dry weight, proline, protein, and GPX enzyme.

Water Stress	Dry Weight (g Pot^{-1})	Total Proline Content (µmol g^{-1} Fresh Weight)	Total Protein Content (mg g^{-1} Fresh Weight)	GPX (µmol H_2O_2. min^{-1} mg^{-1} protein)
Drought	**	**	**	**
100%FC	31.25 ± 0.79 a*	14.33 ± 0.72 c	2.51 ± 0.1 a	0.11 ± 0.01 c
75%FC	26.58 ± 0.83 b	17.15 ± 0.68 b	2.19 ± 0.1 b	0.14 ± 0.02 c
55%FC	18.41 ± 0.42 c	18.49 ± 0.38 a	2.06 ± 0.09 b	0.27 ± 0.02 b
35% FC	11.54 ± 0.51 d	19.28 ± 0.75 a	1.62 ± 0.14 c	0.39 ± 0.03 a
BABA	**	**	*	ns
0(mM)	20.04 ± 2.14 c	14.9 ± 0.81 b	1.85 ± 0.15 b	0.18 ± 0.03
0.8(mM)	20.83 ± 2.23 c	17.76 ± 0.69 a	2.03 ± 0.12 ab	0.23 ± 0.04
1.6(mM)	22.25 ± 2.39 b	17.81 ± 0.84 a	2.18 ± 0.11 a	0.27 ± 0.04
2.4(mM)	24.66 ± 2.48 a	18.77 ± 0.62 a	2.31 ± 0.16 a	0.21 ± 0.04
LSD	1.11	1.39	0.3	0.06

** significant at 0.01 level. * In each row, means with the same letter do not have a significant difference ($p < 0.05$) with each other according to LSD mean comparison test; ns means non-significant.

3.2. Photosynthetic Pigments

Water deficit stress and BABA significantly affected ($p \leq 0.01$) the chlorophylls and carotenoids content in grapefruit mint. The maximum amount of chlorophyll a and b, total chlorophyll, and carotenoids were observed in the control (non-stressed) plants with a foliar application of BABA at 2.4 mM. On the other hand, the lowest amount of all chlorophylls and total carotenoids was observed in the non-elicitor treated plants grown under severe water stress (Table 1). Leaf chlorophyll content is one of the most important physiological characteristics of plants, which usually decreases under drought stress conditions [19]. It has been reported that, during water deficit stress, the production of oxygen radicals increases and, due to peroxidation, the pigments decompose [53]. Therefore, as a result of drought stress, chlorophyll molecules are destroyed and, due to the destruction of pigments, the synthesis of the main complex of chlorophyll pigments is reduced; thus, chloroplast lipids, proteins, and pigments suffer from oxidative damages [54]. Metabolic changes in the cells have been shown

to reduce photosynthetic pigments in sorghum plants under drought stress [55]. Additionally, under drought stress, the amount of carotenoids in corn decreases [56]. Based on our results, BABA application significantly reduced the water stress damages in grapefruit mint. This can be due to the maintenance of the chlorophyll content under BABA application by increasing the cell's antioxidant capacity [8]. Selim et al. [57] reported that BABA treatment improves the photosynthetic pigments in *Medicago intertexta* (L.) Mill. On the other hand, previous studies demonstrated that the pre-treatment of plants with an exogenous GABA elicitor can recover the salinity stress damages and mitigate the negative effects of abiotic stresses in plants [8,58].

3.3. Antioxidant Enzymes Activity

BABA and water deficit significantly ($p < 0.01$) affected APX and SOD activity. However, BABA had no significant effect on the activity of GPX enzymes (Tables 1 and 2). The results demonstrated that the activity of the antioxidant enzymes was up-regulated under water deficit stress. The highest APX and SOD activity was observed in severe drought stress with application of 1.6 mM BABA (Table 1). Interestingly, by increasing the BABA concentration to 2.4 mM, the activity of the APX and SOD enzymes decreased by 13.15 and 15.49% compared with the foliar application of BABA at 1.6 mM under severe stress, respectively. According to the Pearson's correlation analysis, there was a significant and positive relationship among the APX and SOD activity (r = 0.92, $p < 0.01$). On the other hand, there was a significant negative correlation between the activity of the antioxidant enzymes with DW and photosynthetic pigments (Table 3). Antioxidant enzymes play an important role in scavenging reactive oxygen species (ROS) and, under stress conditions, the decrease of lipid peroxidation improves the cell structure and plant growth [59]. Similarly, previous studies on medicinal and aromatic plants have already reported redox regulations under water stress conditions as occurred for *Hyssopus officinalis* L. [60] and *Origanum vulgare* L. [37]. Interestingly, BABA applications at 1.6 mM caused a further increase in the APX and SOD activity under severe water stress conditions. Similarly, Hussain et al. [60] reported that seed priming with BABA confers resistance to drought stress by increasing the antioxidant enzymes (SOD and POX) activities. Ahmadi et al. [60] reported that the foliar application of the amino acid citrulline induces the activation of enzymatic and non-enzymatic antioxidants in hyssop.

3.4. Proline and MDA Content

In grapefruit mint, the proline content was significantly affected by water deficit stress and exogenous BABA applications (Table 2). Thus, under severe drought stress, the total proline content increased by 34.54% compared with the control plants. On the other hand, BABA spraying increased the proline content by about 25.97% compared with the non-treated plants (sprayed with distilled water). The Pearson's correlation analysis showed that proline was positively correlated with the GPX (r = 0.75), APX (r = 0.85), and EO content (r = 0.59) of grapefruit mint. Proline, as an osmolyte and potent antioxidant, plays a key role in plant cells against abiotic stress [58,61]. The accumulation of proline as an osmolyte decreases the stress damage in plants [62]. Proline, by averting enzymatic degradation and eliminating hydroxyl radicals through osmotic regulation, increases the plant tolerance under drought stress conditions [63]. In accordance with our results, Singh et al. [64] reported that BABA treatment under drought stress conditions induces the accumulation of amino acids such as proline in *Arabidopsis*. Similarly, the application of BABA significantly increased the proline content in *M. interexta* sprouts [57]. In our study, as water stress intensified, the proline content increased while the chlorophyll content decreased. Given that glutamate is a common precursor for the synthesis of both chlorophyll and proline, it can be assumed that the increased synthesis of proline under drought stress conditions leads to a decrease in chlorophyll synthesis [65].

Table 3. Pearson's correlation coefficients among grows parameters, physiological characteristics, essential oil content, and compositions of grapefruit mint under different water deficit stress and BABA spraying.

	Fresh Weight	Dry Weight	Chlorophyll a	Chlorophyll b	Total Chloro-phyll	Carotenoid	SOD	Total Proline Content	Total Protein Content	MDA	GPX	APX	Essential Oil Content	Linalool	Dodecane	Linalool Acetate
Fresh Weight	1.00															
Dry Weight	0.99 **	1.00														
Chlorophyll a	0.61 *	0.57 *	1.00													
Chlorophyll b	0.94 **	0.92 **	0.67 **	1.00												
Total Chloro-phyll	0.80 **	0.77 **	0.94 **	0.87 **	1.00											
Carotenoid	0.86 **	0.85 **	0.71 **	0.78 **	0.80 **	1.00										
SOD	−0.76 **	−0.79 **	−0.19	−0.70 **	−0.43	−0.58 *	1.00									
Total Proline Content	−0.56*	−0.61 *	−0.05	−0.51 *	−0.25	−0.39	0.89 **	1.00								
Total Protein Content	0.89 **	0.88 **	0.83 **	0.87 **	0.92 **	0.95 **	−0.55*	−0.37	1.00							
MDA	−0.81 **	−0.80 **	−0.87 **	−0.83 **	−0.93 **	−0.82 **	0.37	0.23	−0.93 **	1.00						
GPX	−0.89 **	−0.89 **	−0.32	−0.85 **	−0.57*	−0.62 **	0.92 **	0.75 **	−0.65 **	0.51*	1.00					
APX	−0.85 **	−0.86 **	−0.29	−0.77 **	−0.52 *	−0.68 **	0.95 **	0.85 **	−0.63 **	0.48	0.931 **	1.00				
Essential Oil Content	−0.15	−0.19	0.20	0.01	0.13	−0.24	0.48	0.59*	−0.09	−0.03	0.20	0.43	1.00			
Linalool	0.64 **	0.64 **	0.37	0.69 **	0.53 *	0.57 *	−0.71 **	−0.54*	0.55*	−0.47	−0.65 **	−0.74 **	−0.38	1.00		
Dodecane	0.59 *	0.63 **	0.23	0.48	0.36	0.73 **	−0.62*	−0.55*	0.63 **	−0.45	−0.48	−0.62 **	−0.64 **	0.54 *	1.00	
Linalool Acetate	−0.72 **	−0.74 **	−0.26	−0.67 **	−0.46	−0.68 **	0.80 **	0.69 **	−0.64 **	0.51*	0.70 **	0.81 **	0.59*	−0.80 **	−0.85 **	1.00

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level. SOD, Superoxide dismutase; GPX, guaiacol peroxidase; APX, ascorbate peroxidase; MDA, malondialdehyde.

The malondialdehyde (MDA) content in grapefruit mint increased as the drought stress intensified (Table 1). The MDA content is used as an index to measure lipid peroxidation in plant tissues (Stewart and Bewley, 1980). The highest values for MDA were observed in severe drought stress with non-BABA spraying, while the lowest amount was observed in the control condition with a foliar application of BABA at 2.4 mM (Table 1). The content of MDA in severe drought stress with the application of BABA (2.4 mM), decreased by about 65% compared with the plants grown under severe drought stress with non-BABA treatment. Our results demonstrated that in all water stress treatments, the MDA content was decreased by increasing the BABA application. The MDA was negatively correlated with fresh and dry weight. Oxidative stress, by increasing membrane lipid peroxidation, produces aldehydes including MDA in plant cells (Morshedloo et al., 2017). Our results were in agreement with earlier reports indicating that BABA treatment notably decreased the MDA content in *Brassica napus* L. seedlings [66] and wheat [67] under stress conditions. BABA may decrease the lipid peroxidation and increase the cell–membrane constancy by increasing the activity of antioxidant enzymes and cell detoxification by ROS scavenging, improving plant tolerance to water deficit stress [57].

3.5. Essential Oil (EO) Content and Compositions

The EO content in grapefruit mint was significantly affected by water stress and the exogenous application of BABA (Figure 1). The EO content varied from 0.22 to 1.17% (*v*/*w*) depending on the BABA treatment and water stress level. The highest amount of EO content was observed in mild drought stress and BABA spraying at 1.6 to 2.4 mM, which increased by about 140% compared with the control condition. On the other hand, the BABA foliar application significantly increased the EO content. So, the foliar application of BABA at 2.4 mM increased the EO content by about 33%, compared with the plants sprayed with distilled water (Figure 1). The GC-MS analysis allowed the identification of 23 compounds

in grapefruit mint EOs, accounting for 96.81–99.53% of the total compositions (Table 4). Among them, linalool (ranging from 33.7 to 47.3%) and linalool acetate (ranging from 31.2 to 52%) were identified as the predominant components in all treatments. The other main components identified were 1,8 cineole, *β*-myrcene, *n*-dodecane, thymol, carvacrol, and geranyl acetate which showed low percentages (Table 4). The foliar application of BABA and different water stress levels significantly affected the linalool, linalool acetate, *β*-myrcene, 1,8-cineole, thymol, carvacrol, and geranyl acetate contents (Figure 1 and Table 4). Linalool acetate was identified as the first dominant compound in grapefruit mint EOs. The foliar application of BABA elicited the production of the main EO component, i.e., linalool acetate.

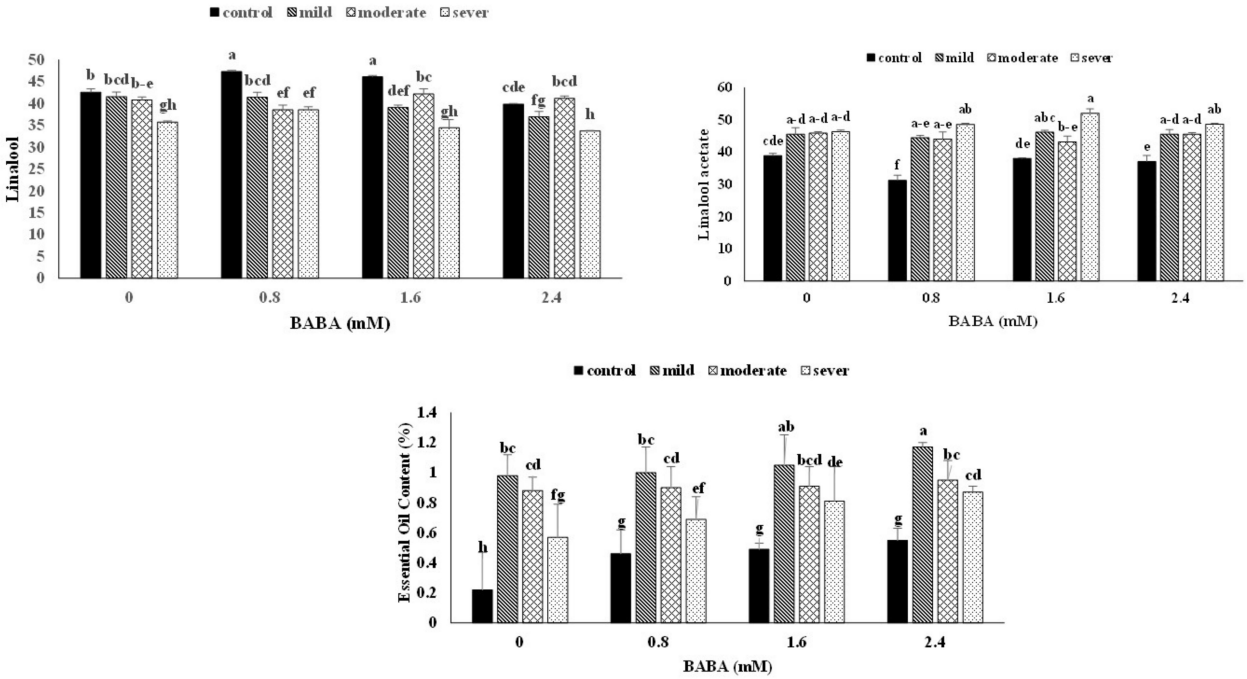

Figure 1. Content of grapefruit mint essential oil, and percentages of linalool, and linalool acetate under different levels of water stress and foliar applications of BABA. Data are means of three independent replications (n = 3). Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences ($p < 0.05$) among the treatments (LSD test at 5% level).

The highest percentage of this component was observed in severe drought stress (35% FC) with a foliar application of BABA at 1.6 mM, which increased by about 34% compared with the control condition. On the other hand, the lowest percentage of linalool acetate was observed in non-stressed plants with a foliar application of BABA at 0.8 mM. Linalool was identified as the second dominant compound in grapefruit mint EOs. The highest percentage of linalool was obtained under normal irrigation (100% FC) with a foliar application of BABA at 0.8 mM, which increased by 11.11% compared with the control condition. The lowest percentage of linalool was observed in severe drought stress with a foliar application of BABA at 2.4 mM, which decreased by 20.71% compared with the control condition (Figure 1). Interestingly, the Pearson's correlation analysis unraveled a positive correlation between the linalool content and dry weight (r = 0.64; $p < 0.01$). On the other hand, there was a significant and negative relationship between the linalool and linalool acetate contents (r = −0.8; $p < 0.01$).

Table 4. Grapefruit mint essential oil compositions under different levels of water stress and BABA applications.

				Treatments (BABA and Water Deficit Stress)															
N.	**Constituents**	**RI**	**RI ***	**0 (mM)**				**0.8 (mM)**				**1.6 (mM)**				**2.4 (mM)**			
35	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**				
1	*n*-Nonane	900	898	0.10	tr.	tr.	-	tr.	-	tr.	tr.	-	-	tr.	-	tr.	-	tr.	-
2	Citronellene	930	929	0.06	-	0.05	0.10	0.06	-	0.07	tr.	-	0.06	-	-	0.05	-	-	0.11
3	β-Pinene	974	972	0.36	0.34	0.425	0.39	0.33	0.32	0.39	0.38	0.26	0.02	0.4	0.195	0.265	0.29	0.41	0.41
4	**1-Octen-3-ol**	**977**	**975**	**0.87 ± 0.02**	**0.60 ± 0.08**	**0.71 ± 0.09**	**0.76 ± 0.04**	**1.16 ± 0.05**	**0.63 ± 0.00**	**0.83 ± 0.07**	**0.69 ± 0.08**	**0.8 ± 0.02**	**0.54 ± 0.1**	**0.80 ± 0.04**	**0.42 ± 0.06**	**1.08 ± 0.03**	**0.60 ± 0.04**	**0.83 ± 0.01**	0.38 ± 0.22
5	**β-Myrcene**	**988**	**988**	**tr.**	**-**	**-**	**2.57 ± 0.26**	**2.31 ± 0.02**	**1.33 ± 0.77**	**2.86 ± 0.12**	**-**	**-**	**2.51 ± 0.18**	**-**	**0.38 ± 0.16**	**-**	**-**	**-**	1.18 ± 0.68
6	*n*-Decane	1000	998	tr.	-	-	-	-	-	-	-	tr.	0.08	-	-	tr.	-	-	-
7	*p*-Cymene	1024	1021	-	tr.	-	-	-	tr.	-	tr.	-	-	-	-	tr.	-	-	-
8	Limonene	1025	1025	-	-	tr.	-	-	-	-	-	-	-	tr.	-	-	-	tr.	-
9	**1,8-Cineole**	**1026**	**1027**	**1.42 ± 0.1**	**1.42 ± 0.06**	**1.71 ± 0.18**	**1.84 ± 0.01**	**1.68 ± 0.03**	**1.42 ± 0.01**	**1.51 ± 0.04**	**1.51 ± 0.09**	**1.43 ± 0.06**	**1.22 ± 0.07**	**1.65 ± 0.05**	**1.33 ± 0.04**	**1.71 ± 0.03**	**1.27 ± 0.09**	**1.65 ± 0.06**	1.52 ± 0.00
10	(*Z*)-β-Ocimene	1032	1035	0.98	0.42	0.40	0.41	0.38	0.38	0.44	0.42	0.37	0.36	0.43	0.37	0.22	0.36	0.38	0.39
11	(*E*)-β-Ocimene	1044	1045	0.65	0.45	0.49	0.49	0.78	0.43	0.52	0.51	0.64	0.39	0.56	0.40	0.91	0.43	0.56	0.54
12	γ-Terpinene	1054	1055	0.11	0.11	0.12	0.17	0.15	0.12	0.15	011	0.09	0.14	0.1	0.09	0.13	0.13	0.11	0.12
13	Terpinolene	1086	1084	0.23	0.19	0.18	0.16	0.30	0.19	0.23	0.17	0.25	0.15	0.21	0.13	0.37	0.175	0.2	0.19
14	**Linalool**	**1096**	**1102**	**42.57 ± 0.76**	**41.58 ± 1.05**	**40.81 ± 0.67**	**35.76 ± 0.23**	**47.30 ± 0.27**	**41.40 ± 1.19**	**38.56 ± 1.08**	**38.52 ± 0.71**	**46.12 ± 0.29**	**39.08 ± 0.57**	**42.18 ± 1.17**	**34.43 ± 1.89**	**39.84 ± 0.17**	**36.95 ± 1.22**	**41.15 ± 0.55**	33.75 ± 0.08
15	*cis*-Pinocamphone	1172	1168	0.81	tr.	-	-	-	-	-	0.07	-	-	tr.	tr.	-	-	-	0.06
16	α-Terpineol	1186	1187	0.37	0.33	0.32	0.30	0.31	0.29	0.28	0.12	0.32	0.29	0.30	0.19	0.40	0.27	0.15	0.41
17	***n*-Dodecane**	**1200**	**1198**	**3.52 ± 0.3**	**2.54 ± 0.29**	**2.31 ± 0.22**	**2.47 ± 0.11**	**4.35 ± 0.36**	**2.38 ± 0.3**	**2.53 ± 0.28**	**2.32 ± 0.19**	**3.7 ± 0.03**	**1.87 ± 0.11**	**3.2 ± 0.27**	**2.19 ± 0.16**	**5.34 ± 0.52**	**2.35 ± 0.05**	**2.59 ± 0.05**	1.37 ± 0.04
18	Nerol	1227	1225	0.65	0.43	0.41	0.29	0.81	0.39	0.43	0.48	0.62	0.39	0.55	0.21	1.07	0.41	0.46	1.52
19	Carvone	1239	1239	-	tr.	-	0.4	-	-	-	-	-	-	tr.	-	-	-	tr.	0.20
20	**Linalool acetate**	**1254**	**1257**	**38.89 ± 0.71**	**45.58 ± 2**	**45.88 ± 0.45**	**46.32 ± 0.51**	**31.28 ± 1.55**	**44.46 ± 0.67**	**44.05 ± 2.17**	**48.58 ± 0.36**	**38.04 ± 0.18**	**46.18 ± 0.59**	**43.13 ± 1.86**	**52.06 ± 1.33**	**37.05 ± 1.88**	**45.49 ± 1.48**	**45.6 ± 0.48**	48.66 ± 0.28
21	**Thymol**	**1289**	**1290**	**1.90 ± 0.22**	**1.18 ± 0.15**	**1.15 ± 0.08**	**1.45 ± 0.13**	**2.345 ± 0.23**	**1.12 ± 0.15**	**1.26 ± 0.14**	**1.25 ± 0.08**	**1.78 ± 0.00**	**0.97 ± 0.08**	**1.55 ± 0.13**	**0.64 ± 0.37**	**3.16 ± 0.38**	**1.22 ± 0.01**	**1.26 ± 0.02**	1.04 ± 0.02
22	**Carvacrol**	**1298**	**1299**	**0.125 ± 0.04**	**0.31 ± 0.02**	**0.29 ± 0.02**	**0.14 ± 0.08**	**0.05 ± 0.00**	**0.26 ± 0.01**	**0.23 ± 0.01**	**-**	**tr.**	**0.26 ± 0.02**	**tr.**	**0.06 ± 0.01**	**0.29 ± 0.02**	**2.58 ± 1.45**	**0.12 ± 0.07**	0.41 ± 0.15
23	Neryl acetate	1361	1363	1.04	0.63	0.70	0.83	1.13	0.62	0.7	0.74	0.97	0.57	0.82	0.66	1.62	1.83	0.75	0.72
24	**Geranyl acetate**	**1381**	**1382**	**2.04 ± 0.22**	**1.20 ± 0.15**	**1.32 ± 0.02**	**1.66 ± 0.13**	**2.19 ± 0.2**	**1.19 ± 0.14**	**1.34 ± 0.14**	**1.41 ± 0.03**	**1.85 ± 0.03**	**1.1 ± 0.03**	**1.57 ± 0.13**	**0.78 ± 0.36**	**3.16 ± 0.4**	**2.55 ± 0.69**	**1.42 ± 0.03**	1.58 ± 0.12
25	(*E*)-Caryophyllene	1417	1413	-	tr.	-	0.21	-	0.06	0.12	tr.	-	0.18	0.02	-	-	tr.	-	0.11
26	(*E*)-β-Farnesene	1454	1454	0.32	0.47	0.43	0.48	0.27	0.46	0.42	0.46	0.34	0.48	0.4	0.53	0.47	1.23	0.39	0.46
27	Bicyclogermacrene	1500	1490	0.63	0.79	0.75	0.68	0.475	0.69	0.66	0.68	0.59	0.74	0.61	1.05	0.89	0.81	0.54	0.90
28	Viridiflorol	1592	1585	0.25	0.34	0.29	0.25	0.34	0.2	0.28	0.25	0.39	0.25	0.23	1.67	0.69	0.17	0.23	0.28

* Linear retention index experimentally determined using a mixture of n-alkanes.

Previous studies on mint species demonstrated that the EO content of grapefruit mint grown in field conditions is about 1.5% (*v*/*w*) which was in accordance with our results [4]. Because of the great importance of EOs in the cosmetic, pharmaceutical, and food industries, in recent years' many studies have focused on their quali-quantitative improvement [68]. EO production in medicinal and aromatic plants depends on different factors including harvesting time, phenological stage, agricultural practice, and biotic and abiotic stresses [13,69]. It seems that during water deficit stress, the production of plant metabolites increases to prevent cell oxidation [70].

Here, under mild and moderate water stress conditions, the EO content of grapefruit mint increased, which may be related to the increase of the glandular trichomes density, as the latter increases with the reduction of the leaf area. Interestingly, leaf and oil gland maturity and the range of stress conditions affect the total content of monoterpene compounds [71]. As depicted in Figure 1, the EO content of grapefruit mint diminished under sever water stress. Similar results have been reported by Ahmadi et al. [60], as they mentioned that severe drought stress significantly decreased the EO production in hyssop plants. It has been shown that under severe drought stress due to the stomata closure, the rate of absorption of CO_2 and subsequently the rate of photosynthesis in plants decreases, which leads to a decrease in the production of secondary metabolites [72]. Similar results have been reported in other medicinal and aromatic plants such as German chamomile [73] and oregano [37]. In fact, according to the intensity of stress and plant species, the amount of EOs could increase, decrease, or remain constant [74]. Govahi et al. [75] reported that the highest EO content in sage is observed in mild drought stress, but its yield decreases. Islam et al. [76] reported that the use of growth regulators leads to a change in EO biosynthesis, leaf area index (LAI), and the number of oil gland structures, all of which affect the EO production. BABA is known as a non-protein amino acid which can improve the plant's

defense mechanism and secondary metabolites under abiotic stress. Our results well concur with the ones of Hafez et al.'s [77] who mentioned that BABA foliar application can increase the EO yield and chamazulene percentage in German chamomile. Some other studies showed the positive role of BABA in the plants' growth and their better response to biotic and abiotic stresses [31]. In this respect, Prins et al. [78] reported that growth regulators change the composition of EOs by changing the biosynthetic pathway of terpenoids and enzymatic processes. Overall, in support of our findings, the investigation of Ahmadi et al. [60] on *H. officinalis* showed that citrulline amino acid at 2 mM elicits drought stress tolerance and increases the *iso*-pinocamphone content.

4. Conclusions

Water deficit stress is one of the main limiting factors affecting mint production in arid and semi-arid regions of the world. Our results showed that water deficit stress reduces the drug yield of grapefruit mint but increases the EO content and composition as it changes the ratio of linalool and linalool acetate. Interestingly, by foliar application of BABA at 1.6 to 2.4 Mm, most of the investigated treats such as EO content and linalool acetate percentage reached the desired value to alleviate water deficit stress. On the other hand, moderate water stress and 1.6 mM BABA gave the highest percentage of linalool acetate (52%) as the main volatile component of grapefruit. Furthermore, the foliar application of BABA improved the antioxidant defense system in grapefruit mint under water stress conditions and significantly reduced the membrane lipid peroxidation and cell damage. Therefore, the application of BABA in the mentioned concentrations can provide the possibility for a high-throughput production of grapefruit mint in regions affected by severe water deficit stress.

Author Contributions: S.A.: investigations, formal data analysis, resources, data curating, visualization and writing. M.R.M.: conceptualization, methodology, supervision, funding acquisition, writing—original draft. F.B.: methodology and data curating, writing—review and editing. H.M.: formal data analysis, resources, data curating, review and editing. F.M.: counselling, methodology, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: The authors have appreciated the central research laboratory of Maragheh University for their kind assistance in the GC-MS and GC-FID analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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