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The Evaluation of Exogenous Application of Salicylic Acid on Physiological Characteristics, Proline and Essential Oil Content of Chamomile (*Matricaria chamomilla* L.) under Normal and Heat Stress Conditions

Mojtaba Ghasemi [1,2], Mohammad Modarresi [2,*], Nadali Babaeian Jelodar [3], Nadali Bagheri [3] and Abbas Jamali [4]

[1] Department of Research and Development, Sea Bioprospecting Co., Ltd., Tangestan Science and Technology Incubator, Persian Gulf Science and Technology Park, Bushehr 7515797414, Bushehr, Iran; m.ghasemi22@gmail.com

[2] Department of Plant Breeding, Faculty of Agriculture and Natural Resources, Persian Gulf University, Bushehr 7561158818, Bushehr, Iran

[3] Department of Plant Breeding and Biotechnology, Faculty of Crop Science, Sari Agricultural Sciences and Natural Resources University, Sari 578, Mazandaran, Iran; n.babaeian@sanru.ac.ir (N.B.J.); n.bagheri@sanru.ac.ir (N.B.)

[4] The Persian Gulf Research and Study Institute, Persian Gulf University, Bushehr 7561158818, Bushehr, Iran; jamalia111@yahoo.com

* Correspondence: modarresi@pgu.ac.ir; Tel.: +98-7734221461

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Abstract: The objective of this study was to investigate the effect of exogenous application of salicylic acid concentrations on the physiological and biochemical traits and essential oil content of chamomile under normal and heat stress conditions as induced by delayed sowing. The experiments were conducted during 2011–2012 as a factorial using a randomized complete block design with three replications, in a very hot region. The factors included five salicylic acid concentrations (0 (control), 1, 10, 25 and 100 mg$\cdot$ L^{-1}) and three chamomile cultivars (Bushehr, Bona, Bodegold). The seeds of chamomile were sown on two different sowing dates including an optimum planting date and a late planting date. The physiological traits (plant height, capitol diameter, 1000 grain weight, fresh and dried flower weight), total chlorophyll, proline and essential oil content were investigated. Analysis of variance showed that the effect of the environmental conditions (normal and heat stress) was significant on all physiological and biochemical traits with the exception of the essential oil content. The heat stress decreased physiological traits and total chlorophyll in comparison with the normal conditions but it had no significant effect on the essential oil content. Findings indicated that the application of exogenous salicylic acid improves essential oil content in chamomile cultivars under environmental heat stress conditions.

Keywords: German chamomile; essential oil; heat stress; salicylic acid; proline; chlorophyll

1. Introduction

Salicylic acid (SA) is a well-known endogenous plant signal molecule involved in biochemical pathways, disease resistance and many plant responses [1]. On the other hand, SA is a signaling molecule that regulates plant responses to heat stress and disease resistance. It can also change to increase heat-induced Hsp/Hsc70 accumulation in higher plants [2,3]. Salicylic acid is necessary for the establishment of systemic acquired resistance in plants [3]. Salicylic acid and jasmonates, through the expression of genes codifying toward enzymes involved in the biosynthesis of secondary metabolites, can increase plant cell productivity [4]. The influence of exogenous salicylic acid depends on various factors, including the concentration of salicylic acid, the developmental phase, the species and the method of application [1]. Lately, the impact of salicylic acid on the metabolic profile of *Catharanthus roseus* cell suspensions has been indicated [5]. Soluble phenolic compounds increase in chamomile plants by using salicylic acid [1]. Salicylic acid relieves the negative effect of oxidative stress and it improves NaCl stress tolerance parameters accompanying mineral nutrient contents in chamomile plants [6]. Chamomile is one of the most crucial medicinal plants in the world which has many utilizations in the food, pharmaceutical, hygienic and cosmetic industries. Botanically, chamomile is an annual plant, belonging to the Asteraceae family and indigenous to Iran, and it grows as a wild plant in Europe [7,8]. Chamomile is naturally dispersed in the south, southwest, west and northwest of Iran and its utilization has a long history in Iranian traditional medicine [9]. Chamomile may be examined as an economical crop for fields with high temperature and water rareness due to its sizeable adaptability to a large spectrum of soils and weather conditions [10,11]. Rowshan and Bahmanzadegan (2013) reported that the the application of exogenous salicylic acid with 200 and 400 $mg\cdot L^{-1}$ concentrations may modify secondary metabolites and their pathway by impacts on plastids, the chlorophyll level and representing stress conditions. The stress produced by SA modifies the quality and quantity of the essential oil of yarrow (*Achillea millefolium*) [12]. The content and compounds of essential oil are different in the chamomile flower and they depend on genotype, and environmental factors such as light intensity, day length, temperature, habitat, management of production and post-harvest processes [13,14]. Variations in oil content and composition have been reported in essential oil–bearing plants such as basil and *Artemisia* under water stress conditions [10,15]. In chamomile, the effects of cropping techniques, planting date, genotypes and ecological conditions on the yield of essential oil and the oil composition have been considered [10,16]. Farhoudi et al. (2012) indicated that medium drought stress increased the oil yield [9]. D'Andrea (2002) reported that there were no statistical differences on the essential oil percentage among the four chamomile cultivars grown in southern Italy [17]. Jeshni et al. (2014) indicated that drought stress caused significant effects on physiological traits, essential oil yield and essential oil components. The essential oil components increased whereas the essential oil yield decreased in response to severe drought stress [8]. In contrast, heat stress has been known as an agricultural problem in many regions in the world. High temperature produces a series of physiological and biochemical modifications in plants, which influence plant growth and development and can lead to severe diminution in economic yield [18–20]. Plants need to acquire thermotolerance against the adverse effect of high temperature. Heat stress damages plant cells and produces osmotic and oxidative stresses [21]. It was shown that the drought stress decreased the physiological parameters and apigenin content in German chamomile but it had no significant effect on the essential oil content [10]. Razmjoo et al. (2008) expressed that increased salinity caused a significant reduction in the plant height, and fresh and dry flower weight of chamomile plants. Also, drought caused a significant reduction in the fresh and dry flower weight and essential oil content of chamomile [15]. The physiological and biochemical traits including proline, photosynthetic pigments (chlorophyll A, B and carotenoids), glutamine synthetase (GS), betaine, ascorbate peroxidase (AOX), soluble proteins, glycine and mineral elements were examined in many plants such as rice, pepper and cabbages under heat stress, salinity, drought and water deficiency conditions [22,23]. For heat tolerance screening in plant breeding programs, the biochemical and physiological parameters were evolved efficiently under heat stress or drought conditions [19]. Heidari and Sarani (2012) reported that the proline and soluble carbohydrates increased in chamomile leaves under iron deficit and salinity treatments [22]. The proline level and antioxidative activity of Indian mustard (*Brassica juncea* L.) reduced significantly in response to salicylic acid under heat stress, but the plants subjected to high temperature showed a significant reduction in growth, photosynthesis traits and chlorophyll content [24]. The purpose of this investigation was to examine the influence of the exogenous application of salicylic acid concentrations on the physiological and biochemical parameters and essential oil content of chamomile (*Matricaria chamomilla* L.) under normal and heat stress conditions.

2. Material and Methods

2.1. Experimental Site Description

Two factorial field experiments were performed using a randomized complete block design (RCBD) with three replications at the experimental farm of Bushehr Research Center for Agriculture and Natural Resources, Borazjan, Iran, during the 2011–2012 season. The geographical coordinates of the experiment site was 29°12′21″ N, 51°15′07″ E, with altitude of 110 m. The chemical and physical properties of soil and water of the experimental location are presented in Tables 1–3. The chamomile seeds were provided by the seed bank of the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. Each experimental plot size was 1 m × 1 m and in each plot, the plants were grown in three equidistant rows with adjacent rows being 30 cm apart. In each plot, 20 g of ammonium nitrate fertilizer was used before planting date in addition to another 20 g being applied one month hence. Each experimental site had 45 plots (including 15 plots in each block) and totally the two experiments had 90 plots. The distance between the two main plots or experimental sites was seven meters. The seeds were sown superficially by hand and then were covered through a very thin layer of sandy soil.

Table 1. Chemical characteristics for soil of experimental field before implementation of experiment.

Soil Depth (cm)	EC ($ds \cdot m^{-1}$)	pH	T.N.V * (%)	O.C ** (%)	$Ca^{2+} + Mg^{2+}$	Na^+	Cl^-	HCO_3^-	SO_4^-	SAR
					meq/L					
0–30	6.3	7.8	60	0.33	58	25	20	4	59	4.6

* T.N.V: Total Neutralizing Value, ** O.C: Organic Carbon.

Table 2. Chemical properties for irrigation water of experiment.

EC ($ds \cdot m^{-1}$)	pH	HCO_3^-	Cl^-	SO_4^-	$Ca^{2+} + Mg^{2+}$	Na^+	SAR
		$meq \cdot L^{-1}$					
3.7	7.6	4.5	8.5	36	37	12	2.8

Table 3. Physical characteristics for soil of experimental field before implementation of experiment.

Soil Depth (cm)	Bulk Density $g \cdot cm^{-1}$	Humidity (%/w) in F.C Status	Humidity (%/w) in P.W.P Status	Particle Density $g \cdot cm^{-1}$	The Average of Penetration Rate ($cm \cdot h^{-1}$)	Moment Penetration Rate ($cm \cdot h^{-1}$)	Saturation Percent	Texture	Clay Percent
0–30	1.26	16.9	6.3	2.65	10.5	6.2	32	S.L.	12
30–60	1.43	15.4	6.5	2.65					
60–90	1.43	13.3	7.2	2.65					

2.2. Plants Growth and Air Temperature

To evaluate the impact of high temperature (heat stress) on growth, development and yield of plants under field conditions, several different locations in the hot zones, different sowing dates in hot areas and/or controllable growth chambers [25–29] are utilized. Hence, in this study, high temperature treatment was conducted under field (real) conditions in a very hot area in the southwest of Iran by changing sowing date (late planting date). The seeds of chamomile cultivars (Bushehr (diploid), Bona (diploid) and Bodegold (tetraploid)) were planted on two different sowing dates corresponding to optimum planting date (24 December 2011) and late planting date (7 February 2012). The late sowing date (as the heat stress treatment) was set up so that more vegetative stages and complete flowering period were meet with high temperature at the end of agronomic season in Bushehr province. The meteorological conditions during the experimental year (2011–2012) for growth and development of chamomile are presented in Table 4.

Table 4. Meteorological data during the experimental year (2011–2012) for growth and development of chamomile in Borazjan, Bushehr Province, Iran.

Month and Year	Average of Temperature (°C)		Average of Relative Humidity (%)		Precipitation (mm)	Average of Sunny Hours (h)	Evaporation (mm)
	Min	Max	Min	Max			
November 2011	17.8	30.6	27	60	39.4	7.4	5.3
December 2011	10.2	21.4	45	82	62.2	8	2.9
January 2012	10.6	22	42	76	32.1	6.7	2.9
February 2012	10.1	21	39	77	33.8	5.7	3.4
March 2012	10.9	24.1	29	69	22.1	6.8	5.2
April 2012	18.1	31.8	21	64	10.2	6.6	8.8
May 2012	25.7	40.6	12.2	34	0	7.3	11.3
June 2012	28.2	44.1	13	36	0	10	14.2

2.3. Salicylic Acid Treatments

Salicylic acid concentrations including five levels (0 (control), 1, 10, 25 and 100 mg$\cdot$ L^{-1}) were prepared and used on three German chamomile cultivars (Bushehr (diploid), Bona (diploid) and Bodegold (tetraploid). The salicylic acid was purchased from Merck Co. (Darmstadt, Germany). Foliar spray using salicylic acid was scheduled during the growing and flowering stages of chamomile plants. The salicylic acid treatments were applied three times during vegetative and reproductive phases at every 15 days. The first stage of salicylic acid foliar spray was at 60 days after planting. The spray was done at 10 a.m. until 2 p.m. for each treatment and plot. All spray solutions were applied to the shoots uniformly using a hand pump sprayer. Precise dates of foliar spraying using salicylic acid solutions are presented in Table 5.

Table 5. Spraying dates of chamomile by salicylic acid under normal and heat stress conditions.

Time of Spraying	Environmental Conditions	
	Normal	Heat Stress
1	21 February 2012	8 April 2012
2	8 March 2012	23 April 2012
3	22 March 2012	8 May 2012

2.4. Physiological Characteristics

The evaluated physiological characters included plant height (cm), capitol diameter (mm), 1000 grain weight (g), fresh flower weight (g) and dried flower weight (g). The plant height was calculated at full bloom period as a mean height of 10 plants in each plot using meter. After each harvest samples were weighed (±0.001 g) using balance set and after drying at room temperature (20–25 °C) dried flower weight of each plot was calculated. The flower capitol diameter was measured at full bloom period as a mean capitol diameter of 15 flowers in each plot by using digital caliper. The 1000 grain weight trait was calculated using seed counter device and digital balance. The chamomile flowering was initiated in early March 2012 and it continued until end of May 2012. Thus, the harvest was carried out every seven to 10 days for each plot. The number of harvests and its dates are presented in the Table 6.

Table 6. Harvest times of chamomile cultivars under normal and heat stress conditions.

	Environmental Conditions					
	Normal			Heat Stress		
Harvest Time	Bushehr Cultivar	Bona Cultivar	Bodegold Cultivar	Bushehr Cultivar	Bona Cultivar	Bodegold Cultivar
1	27 Mar 2012	19 Apr 2012	28 Apr 2012	22 Apr 2012	04 May 2012	31 May 2012
2	07 Apr 2012	29 Apr 2012	08 May 2012	03 May 2012	14 May 2012	-
3	19 Apr 2012	08 May 2012	14 May 2012	13 May 2012	31 May 2012	-
4	29 Apr 2012	14 May 2012	-	31 May 2012	-	-
5	14 May 2012	-	-	-	-	-

2.5. Chlorophyll Measurement

In order to estimate the total chlorophyll, leaf samples were collected and dried for 48 h at a temperature of 75 °C. The amount of chlorophyll was estimated according to the method of Lichtenthaler et al. (1987) [30].

2.6. Determination of Free Proline Concentration

Free proline content was determined according to a modified method of Bates et al. (1973) [31], in dried leaf samples which were homogenized in 5 mL of sulphosalycylic acid (3%) by using mortar and pestle. To 2 mL of extract in a test tube, 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent were added. The mixture was boiled in a water bath at 100 °C for 30 min and then allowed to cool. After that, 6 mL of toluene were added and the combination transferred to a separating funnel. After thorough mixing, the chromophore-containing toluene was separated and the absorbance read at 520 nm in a spectrophotometer against a toluene blank. Finally, the concentration of proline was calculated using a standard curve [22,32].

2.7. Extraction of Essential Oil

The essential oil of air-dried flower of chamomile (30 g) was isolated by hydrodistillation for 5 h, using a Clevenger-type apparatus in 500 mL round-bottom flask with 300 mL distillated water according to the method described in British Pharmacopeia [10]. The essential oil was stored in dark glass bottles and then dried over anhydrous sodium sulphate (Na_2SO_4). Finally, the essential oil was kept in refrigerator (4 °C) until they were analyzed [33].

2.8. Data Analysis

After physiological and biochemical evaluation, statistical analyses were performed by using MSTAT-C and SAS System Software version 6.12 [34]. Data were analyzed by combined analysis of variance and the mean results were compared by using Duncan's multiple range test. Data were converted when necessary before analysis to satisfy suppositions of normality and homoscedasticity.

3. Results and Discussion

3.1. Plant Height

The analysis of variance showed that the plant height was significantly influenced by environmental conditions and chamomile cultivars (Table 7). The interaction between environmental conditions and cultivars was significant (Table 8), while the effect of SA treatment was not significant on the plant height trait. Duncan analysis for the interaction between cultivars and environmental conditions showed that the Bodegold cultivar had the highest plant height with an average of 64.41 cm under normal conditions. In contrast, the Bushehr cultivar had the lowest plant height with an average of 22.01 cm under heat stress conditions. Meanwhile, the Bona cultivar with a height average of 60.04 cm in comparison with the Bodegold cultivar had no significant differences under normal conditions (Table 8). The Bodegold and Bona cultivars had the greatest plant height with averages of 64.41 cm and 60.04 cm, respectively. These cultivars showed 35.9 cm and 12.34 cm reductions, respectively, in the plant height trait under heat stress conditions, while the Bushehr cultivar underwent a 12.07 cm reduction in the plant height trait under the same conditions. D'Andrea (2002) showed that the tetraploid cultivars of German chamomile in comparison with the diploid cultivars had greater plant height [17]. Alexandra (2005) reported that the Bona cultivar in comparison with the Goral and Lutea cultivars had a higher plant height [35]. Baghalian et al. (2010) reported that drought stress decreased the chamomile plant height [10]. Farhoudi et al. (2012) illustrated that severe drought caused substantial reductions in the plant height of chamomile [9]. Razmjoo et al. (2008) indicated that drought stress caused a significant reduction in the plant height of chamomile [15]. The reduction in plant height seems to be the result of disturbed plant water relations, in particular the turgor potential [36]. This means that reduced water uptake results in a decrease in the tissue water contents and turgor. Therefore, under heat stress conditions, cell elongation in plants is inhibited by reduced turgor pressure. In addition, heat stress also trims down the photo-assimilation and required metabolites for cell division. As a consequence, impaired mitosis, cell elongation and expansion result in reduced plant height and growth [10,37]. These findings showed that the chamomile tetraploid cultivar had a higher plant height under normal conditions in comparison with the diploid cultivars. Also, the severe heat stress reduced the plant height in the tetraploid cultivar in particular. This is in accordance with results of D'Andrea (2002) [17].

3.2. Capitol Diameter

The analysis of variance indicated that the capitol diameter trait was significantly influenced by environmental conditions and the cultivar at the statistical level ($p \leqslant 0.01$) (Table 7). The interaction between environmental conditions and cultivars also was significant at the statistical level ($p \leqslant 0.01$). The mean comparison for the interaction between environmental conditions and cultivars showed that the Bodegold cultivar had the highest capitol diameter with an average of 8.94 mm under normal conditions. In contrast, the Bushehr cultivar had the lowest capitol diameter with an average of 6.43 mm under normal conditions, although the Bodegold cultivar showed a 2.08 mm reduction in the capitol diameter trait under heat stress conditions (Table 8). Alexander (2005), in an investigation on the morphological and chemical characteristics of chamomile, reported that the highest capitol diameter belonged to tetraploid cultivars [35]. Circella et al. (1993), in a study on the morphological traits of different genotypes of chamomile in southern Italy, observed that the tetraploid cultivars had the highest capitol diameter [38]. D'Andrea (2002), in research on the diversity of four tetraploid and diploid cultivars based on morphological characteristics, reported the highest capitol diameter for tetraploid cultivars in southern Italy [17]. Razmjoo et al. (2008) found that increasing the salinity stress caused a reduction in the number of branches per plant, the head diameter, the peduncle length and the flowers per plant. Also, drought caused a significant reduction in the head diameter [15]. In the present research, the capitol diameter of the tetraploid cultivar was greater than the diploid cultivars and also it was reduced under the heat stress conditions in contrast with the normal conditions. Therefore, these results confirm previous research regarding the negative effect of abiotic stress on morphological traits in plants.

3.3. The 1000 Grain Weight

The analysis of variance demonstrated that the 1000 grain weight was significantly influenced by the environmental conditions and the interaction of the environmental conditions and cultivars at the statistical level ($p \leqslant 0.01$) (Table 7). The mean comparison of the interaction between environmental conditions and cultivars indicated that the Bona cultivar had the highest 1000 grain weight with an average of 0.056 g under normal conditions. In contrast, the Bona cultivar had the lowest 1000 grain weight with an average of 0.035 g under heat stress conditions (Table 8). The Bona cultivar showed a 0.021 g reduction in the 1000 grain weight under severe heat stress. Modarresi et al. (2010)

reported that the 1000 grain weight decreased in wheat under heat stress in comparison with normal conditions [26]. In a similar experiment, Mohammadi et al. (2007) demonstrated that the 1000 grain weight diminished in wheat as a consequence of heat stress under controlled greenhouse conditions [25]. Ayeneh et al. (2002) showed that the 1000 grain weight decreased extremely in wheat under natural heat stress at the end of the agronomic season [28]. The main reason for the reduction of the 1000 grain weight in chamomile plants may be attributed to genotypes and the effect of heat stress during grain filling [28].

3.4. Fresh Flower Weight

The analysis of variance showed that the fresh flower weight was significantly influenced by the interaction between the environmental conditions and cultivars at the statistical level ($p \leqslant 0.01$). The fresh flower weight was also significantly influenced by the interactions of the environmental conditions × cultivars and the environmental conditions × salicylic acid (Table 7). Duncan analysis for the interaction between environmental conditions and cultivars showed that the Bona cultivar produced the highest fresh flower weight with an average of 260.42 $g{\cdot}m^{-2}$ under normal conditions. In contrast, the Bodegold cultivar produced the lowest fresh flower weight with an average of 11.85 $g{\cdot}m^{-2}$ under heat stress conditions (Table 8). The mean comparison for the interaction between environmental conditions and salicylic acid indicated that the highest fresh flower weight was obtained with an average of 287.97 $g{\cdot}m^{-2}$ at the concentration of 1 $mg{\cdot}L^{-1}$ SA under normal conditions. In contrast, the lowest fresh flower weight reached was an average of 83.09 $g{\cdot}m^{-2}$ at the concentration of 25 $mg{\cdot}L^{-1}$ SA under heat stress conditions (Table 9). Razmjoo et al. (2008) reported that increased salinity caused a significant reduction in the fresh and dry flower weight. Also, drought caused a significant reduction in the fresh and dry flower weight [15]. On the other hand, Baghalian et al. (2010) reported that drought stress decreased the flower yield and shoot weight [10]. D'Andrea (2002) reported that the chamomile diploid cultivars had the highest dried and fresh flower weight [17]. Circella et al. (1993) observed that the chamomile diploid cultivars had the greatest flower yield and 100 flower weight [38]. In the present research, the Bona cultivar (diploid) produced the greatest dried and fresh flower weight under normal and heat stress conditions, which is in a good agreement with the results of D'Andrea (2002) and Golparvar et al. (2011) [17,39].

Table 7. Combined analysis of variance for studied traits of German chamomile.

		Mean Square (MS)							
S.O.V	**DF**	**Plant Height (cm)**	**Capitol Diameter (mm)**	**Fresh Flower Weight (g)**	**Dried Flower Weight (g)**	**1000 Grain Weight (g)**	**Total Chlorophyll (μg/g· dw)**	**Proline (μmol/g· dw)**	**Essential Oil% (*w*/*w*)**
EC	1	9098.47 **	10.51 **	241064.95 **	14175.22 **	0.0024 **	19337.71 **	15090.63 **	0.0008 ns
Block	4	19.98	0.04	6602.1	328.69	0.00015	18.32	165.45	0.0001
Cult	2	5303.06 **	9.22 **	171402.77 **	9794.01 **	0.00022 ns	130.21 **	3423.70 **	0.0006 ns
SA	4	68.18 ns	0.24 ns	18051.77 *	1080.04 **	0.00008 ns	10.61 ns	116.75 ns	0.0004 ns
Cult × SA	8	12.66 ns	0.10 ns	1750.12 ns	71.85 ns	0.0001 ns	32.82 ns	222.09 *	0.0001 ns
EC × Cult	2	1406.06 **	15.29 **	34884.14 **	1031.30 *	0.00064 **	29.77 ns	9070.7 **	0.0023 **
EC × SA	4	89.27 ns	0.15 ns	28412.84 **	1124.1 **	0.0001 ns	1.46 ns	10.60 ns	0.0002 ns
EC × Cult × SA	8	29.75 ns	0.17 ns	6359.83 ns	323.79 ns	0.0001 ns	9.26 ns	216.25 *	0.0001 ns
Error	56	35.41	6.79	6656.86	290.1	0.000085	23.91	105.41	0.0003
C.V		13.9	4.8	51.9	45.06	18.9	24.2	34.7	19.7

EC (environmental conditions), Cult (cultivar), SA (salicylic acid), symbol (*) and (**): indicates statistically significant differences between treatments at ($p \leqslant 0.05$) and ($p \leqslant 0.01$) levels, respectively, as well as the letters (ns): shows statistically non-significant differences between treatments.

Table 8. Mean comparison of the interaction of environmental conditions × cultivar on studied characteristics of German chamomile.

Characteristics								
Environmental Conditions	**Cultivar**	**Plant Height (cm)**	**Capitol Diameter (mm)**	**Fresh Flower Weight (gr· m^{-2})**	**Dried Flower Weight (gr· m^{-2})**	**1000 Grain Weight (g)**	**Proline (μmol/g· dw)**	**Essential Oil % (*w*/*w*)**
Normal	Bushehr	34.08c	7.18b	181.75ab	42.06ab	0.052a	24.76b	0.77d
	Bona	60.04a	6.82bc	260.42a	65.63a	0.056a	10.96c	0.82c
	Bodegold	64.41a	8.94a	184.42ab	43.33ab	0.054a	14.26c	1.23a
Heat stress	Bushehr	22.01d	6.43c	79.99bc	19.05bc	0.049ab	15.80c	0.89b
	Bona	47.70b	7.59ab	224.22ab	51.08a	0.035b	71.56a	0.76de
	Bodegold	28.49cd	6.86bc	11.85c	5.60c	0.046ab	40.31b	0.74e

3.5. Dried Flower Weight

The analysis of variance indicated that the dried flower weight was significantly influenced by the environmental conditions, salicylic acid and cultivar ($p \leqslant 0.01$) (Table 7). The interaction between the environmental conditions and cultivars had a significant effect on the dried flower weight ($p \leqslant 0.05$). In addition, the interaction between the environmental conditions and the salicylic acid had a significant effect on the dried flower weight ($p \leqslant 0.01$) (Table 7). The means comparison of the interaction between the environmental conditions and cultivars showed that the Bona cultivar had the highest dried flower yield with averages of 65.63 and 51.08 $g \cdot m^{-2}$ under normal and heat stress conditions, respectively. Also, the Bodegold cultivar had the lowest dried flower yield with an average of 5.60 $g \cdot m^{-2}$ under heat stress conditions (Table 8). Duncan analysis for the interaction between the environmental conditions and salicylic acid showed that the highest dried flower weight obtained was an average of 67.66 $g \cdot m^{-2}$ at the concentration of 1 $mg \cdot L^{-1}$ SA under normal conditions. In contrast, the lowest dried flower weight obtained was an average of 21.45 $g \cdot m^{-2}$ at the concentration of 25 $mg \cdot L^{-1}$ SA under heat stress conditions (Table 9). Taviana (2001) and D'Andrea (2002) reported the highest dried and fresh flower yields in chamomile diploid cultivars [17,40]. Farhoudi et al. (2012) showed that severe drought caused substantial reductions in the shoot and flower dry weight of chamomile [9]. Razmjoo et al. (2008) indicated that increased salinity and drought stress caused a significant reduction in the fresh and dry flower weight [15]. In this study, the Bona cultivar (diploid) had the highest dried flower weight which is in accordance with the results of D'Andrea (2002) and Golparvar et al. (2011) [17,39].

Table 9. Mean comparison for the interaction between environmental conditions and salicylic acid on studied traits of chamomile.

		Traits	
Environmental Conditions	**SA Concentration ($mg \cdot L^{-1}$)**	**Fresh Flower Weight ($g \cdot m^{-2}$)**	**Dried Flower Weight ($g \cdot m^{-2}$)**
Normal	0	122.42bc	30.82b
	1	287.97a	67.66a
	10	188.53abc	45.67ab
	25	176.31ab	43.41ab
	100	269.09ab	64.14a
Heat Stress	0	140.10abc	29.24b
	1	107.64c	27.20b
	10	96.86c	23.18b
	25	83.09c	21.45b
	100	99.09c	25.13b

3.6. Total Chlorophyll Content

The analysis of variance showed that the total chlorophyll content was significantly affected by the environmental conditions and cultivars ($p \leqslant 0.01$) (Table 7). The mean comparison for the total chlorophyll content was performed between two environmental conditions (normal and heat stress). A significant reduction in the total chlorophyll content was observed under heat stress in comparison with normal conditions. The total chlorophyll content averaged 34.90 $\mu g/g \cdot dw$ under normal conditions. In contrast, the content of the total chlorophyll attained was an average of 5.59 $\mu g/g \cdot dw$ under heat stress conditions. In fact, severe heat stress caused a 29.31 $\mu g/g \cdot dw$ reduction in the total chlorophyll content under heat stress conditions (Figure 1). The chlorophyll content declined under heat stress in comparison with normal conditions [41]. The reduction of chlorophyll content is one of the most important and effective factors in the plant photosynthetic capacity [42]. Therefore, the decrease of the vegetative characterizations is related to the reduction of the photosynthesis pigment capacity. El-Khallal et al. (2009) reported that the content of photosynthetic pigments (chlorophyll A and B, carotenoids and total pigments) was reduced significantly in leaves of stressed plants and was increased in brassinolide and salicylic acid–treated plants [43]. The application of SA prevents chlorophyll degradation in grapevine leaves under heat stress [44]. Rowshan and Bahmanzadegan (2013) reported that the application of exogenous salicylic acid with 200 and 400 mg· L^{-1} concentrations may modify secondary metabolites of yarrow (*Achillea millefolium*) and its pathway by effects on plastids, the chlorophyll level and representing stress conditions [12]. Farhoudi et al. (2012) reported that severe drought caused significant reductions in chlorophyll A contents and on the photosynthesis rate of chamomile [9]. A number of reports illustrated that chlorophyll biosynthesis was reduced in plants subjected to high-temperature stress [45,46]. Lesser accumulation of chlorophyll in heat-stressed plants may be attributed to impaired chlorophyll synthesis or its accelerated degradation or a combination of both effects. The inhibition of chlorophyll biosynthesis under high-temperature regimes results from a destruction of numerous enzymes involved in the mechanism of chlorophyll biosynthesis [47]. As stated in the Introduction, the influence of exogenous salicylic acid depends on various factors, including the concentration of salicylic acid, the developmental phase, the species and the method of application [1]. The lack of effect of the salicylic acid on the chlorophyll content may be due to the high intensity of heat stress so that severe heat stress impairs plastid and chlorophyll structures and decreases the contributing effect of exogenous salicylic acid. Also, it can be attributed to different responses of chamomile cultivars to specific concentrations and/or certain ranges of exogenous salicylic acid. In addition, the lack of salicylic acid effects on the chlorophyll content can be associated with time and the stage of sampling for chlorophyll measurement in the field conditions.

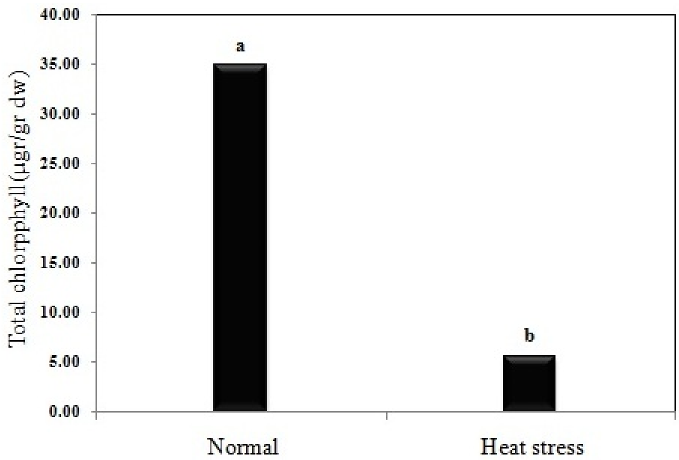

Figure 1. Comparison of the simple effect of normal and heat stress conditions on the total chlorophyll content in chamomile dried leaves.

3.7. Free Proline Concentration

The analysis of variance showed that the free proline concentration was significantly influenced by the environmental conditions and cultivars ($p \leqslant 0.01$). The interaction of cultivar × salicylic acid and the triple interaction of environmental conditions × cultivar × salicylic acid had a significant effect on the free proline content ($p \leqslant 0.05$). Also, the interaction of environmental conditions × cultivar had a significant effect on the proline content ($p \leqslant 0.05$) (Table 7). The mean comparison for the interaction of environmental conditions × cultivar showed that the Bona cultivar had the highest leaf free proline content with an average of 71.56 µmol/g· dw under heat stress conditions. In contrast, the lowest amount of leaf free proline content belonged to the Bona cultivar with an average of 10.96 µmol/g· dw under normal conditions (Table 8). Duncan analysis for the triple interaction of environmental conditions × cultivar × salicylic acid on leaf free proline content indicated that the Bona cultivar had the highest amount of free proline content with an average of 91.96 µmol/g· dw in the control treatment under heat stress, whereas the Bona cultivar had the lowest amount of free proline content with an average of 7.96 µmol/g· dw at the concentration of 25 mg· L^{-1} SA under normal conditions (Table 10).

Also, the Bona cultivar had the highest free proline content in comparison with the Bushehr and Bodegold cultivars with averages of 64.40, 67.70, 75.40 and 58.36 μmol/g· dw at the concentrations of 1, 10, 25 and 100 mg· L^{-1} SA under heat stress, respectively. In contrast, the lowest free proline content belonged to the Bona cultivar with an average of 9.53, 7.96 and 10.06 μmol/g· dw at the concentration of 10, 25 and 100 mg· L^{-1} SA under normal conditions, respectively (Table 10). Kumar et al. (2012) reported that the proline content increased in wheat under heat stress conditions [32]. The antioxidative activity and proline level of Indian mustard (*Brassica juncea* L.) decreased significantly in response to salicylic acid under heat stress, but the plants subjected to high temperatures showed a significant reduction in growth, photosynthesis traits and chlorophyll [24]. Jeshni et al. (2014) showed that the proline content of chamomile increased in response to severe drought stress [8]. Farhoudi et al. (2012) showed that the proline, soluble carbohydrates and malondialdehyde contents of chamomile were increased under drought stress [9]. The proline is the most important substance to regulate the osmotic tension in higher plants under abiotic stresses such as salinity, drought, heat and cold [31]. The increase of the proline concentration is due to extensive protein degradation under abiotic stress such as drought (water deficit) and heat stress [19,48].

Table 10. Mean comparison of the interaction of environmental conditions × cultivar × salicylic acid on proline content according to (μmol/g· dw) of chamomile.

		SA Concentration (mg· L^{-1})				
Environmental conditions	Cultivar	0	1	10	25	100
Normal	Bushehr	34.73b	29.30b	20.30c	20.06c	26.56bc
	Bona	13.13c	14.23b	9.53c	7.96c	10.06c
	Bodegold	11.70c	11.93b	16.13c	13.76c	17.80c
Heat Stress	Bushehr	13.30c	22.96b	18.90c	11.70c	12.16c
	Bona	91.96a	64.40a	67.70a	75.40a	58.36a
	Bodegold	27.36bc	47.80a	41.50b	42.06b	42.83ab

3.8. Essential Oil Content

The analysis of variance showed that the essential oil content was significantly influenced by the interaction between environmental conditions and cultivars ($p \leqslant 0.01$) (Table 7). The mean comparison of the interaction between environmental conditions and cultivars indicated that the Bodegold cultivar had the highest and lowest essential oil content with averages of 1.23% (*w*/*w*) and 0.74% (*w*/*w*) under normal and heat stress conditions, respectively (Table 8). In addition, a mean comparison was carried out for essential oil content under normal and heat stress conditions separately (Tables 11 and 12). The results of the mean comparison for each experimental site separately showed that the highest essential oil content with an average of 0.126% (*w*/*w*) was found in the Bona cultivar at a concentration of 25 mg· L^{-1} SA under normal conditions. In contrast, the lowest essential oil content with an average of 0.076% (*w*/*w*) was found in the Bushehr and Bodegold cultivars at concentrations of 0 and 1 mg· L^{-1} SA under normal conditions, respectively (Table 11). The mean comparison for essential oil content under heat stress conditions showed no significant differences (Table 12). Biosynthesis of secondary metabolites is not only controlled genetically but is also strongly affected by environmental parameters [10,16,49]. Salamon (2006) reported that the essential oil content was 0.24% to 2.0% in chamomile dried flowers and its essential oil color was blue to dark blue [14]. The essential oil extracted had a blue to dark blue color in the present study. This is may be related to the increase in secondary metabolites such as phenolics including flavonoids, anthocyanins, and plant steroids which are also significantly involved in plant responses under heat stress [19,50]. The compositions of essential oil were compared in Egyptian and Brazilian chamomile ecotypes. The essential oil percent was reported from 0.2% to 0.3% in Brazilian ecotypes and it was also reported at 0.5% in Egyptian chamomile ecotypes [51]. Rowshan and Bahmanzadegan (2013) reported that the the application of exogenous salicylic acid with 200 and 400 mg· L^{-1} concentrations may modify secondary metabolites and their pathway by impacts on plastids, the chlorophyll level and representing stress conditions. The salicylic acid, like stress, manipulated the quality and quantity of the essential oil of yarrow (*Achillea millefolium*) [12]. D'Andrea (2002) reported that there were no statistical differences between the essential oil percentage among the four chamomile cultivars grown in southern Italy [17]. Jeshni et al. (2014) indicated that drought stress caused significant effects on physiological traits, essential oil yield and essential oil components. The essential oil components increased, whereas the essential oil yield decreased in response to severe drought stress [8]. Baghalian et al. (2010) indicated that drought stress had no significant effect on the oil content or oil composition [10]. Razmjoo et al. (2008) showed that the increased salinity and drought stress caused a significant reduction in the essential oil content [15]. The reduction in the essential oil content may be due to a disturbance in photosynthesis and carbohydrate production under stress conditions and suppression of the plant growth [15,52]. Changes in oil content and composition are reported in essential oil–bearing plants such as basil and *Artemisia* under water stress conditions [10,15]. Farhoudi et al. (2012) indicated that medium drought stress increased the oil yield [9]. In chamomile, the effects of cropping techniques, planting date, genotypes and ecological conditions on the yield of essential oil and oil composition have been considered [10,16]. Variation in essential oil content and composition in Iran is ascribed to the influence of agricultural practices, and environmental and genetic factors [53]. The essential oil content in chamomile plants depends on three groups of factors: genotype, weather, and agrotechnical factors [54]. The lack of the effect of salicylic acid on the essential oil content may be due to plant species and genotypes that have different responses to salicylic acid under environmental conditions. Moreover, it can be ascribed to changes in the secondary metabolites pathway by effects on the plastids and chlorophyll level under severe heat stress. Furthermore, it may be due to different responses of chamomile genotypes to specific concentrations and/or certain ranges of exogenous salicylic acid. It is probable that the salicylic acid concentrations applied in this experiment were not efficient against the acute heat stress. Generally, salicylic acid can influence the quality of chamomile essential oil.

Table 11. Mean comparison for essential oil% (*w*/*w*) of chamomile treated with salicylic acid under normal conditions.

Cultivar	SA Concentration (mg·L^{-1})				
	0	1	10	25	100
Bushehr	0.076b	0.103ab	0.086b	0.100ab	0.103ab
Bona	0.100ab	0.093ab	0.093ab	0.126a	0.090b
Bodegold	0.086b	0.076b	0.093ab	0.096ab	0.096ab

Table 12. Mean comparison for essential oil% (*w*/*w*) of chamomile treated with salicylic acid under heat stress conditions.

Cultivar	SA Concentration (mg·L^{-1})				
	0	1	10	25	100
Bushehr	0.090a	0.090a	0.080a	0.100a	0.086a
Bona	0.086a	0.090a	0.080a	0.096a	0.100a
Bodegold	0.076a	0.083a	0.080a	0.093a	0.100a

4. Conclusions

In summary, the results of this experiment illustrated that heat stress caused a significant reduction in the physiological characteristics (including plant height, capitol diameter, fresh flower weight and dried flower weight) and chlorophyll content, but it had no significant effect on the essential oil content. However, the proline content increased in response to heat stress. The lack of effect of the salicylic acid on the chlorophyll and essential oil content may be due to plant species, genotype and the high intensity of heat stress. Severe heat stress changes the secondary metabolites pathway and impairs plastid and chlorophyll structures. Also, severe heat stress reduced the improving effect of salicylic acid in the field conditions. In addition, it can be attributed to different responses of chamomile genotypes to specific concentrations and/or certain ranges of exogenous salicylic acid. Nevertheless, the response of chamomile cultivars was different to salicylic acid concentrations under normal and heat stress conditions. Regarding these results, chamomile can be proposed as a moderate heat-resistant medicinal plant with an admissible yield. Findings of this research may support the positive effect of plant growth regulators such as salicylic acid on the amelioration of quality and quantity of chamomile essential oil content under normal abiotic stress such as heat stress. Furthermore, it seems that more investigations are required in order to elucidate physiological and biochemical mechanisms of heat tolerance in German chamomile.

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