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Physiological, Biochemical, and Agronomic Trait Responses of *Nigella sativa* Genotypes to Water Stress

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Abstract: Water stress may affect the growth, physiology, morphology, biochemistry, and productivity of *Nigella sativa* (black cumin), a medicinal and aromatic plant. Measuring these parameters under various irrigation regimes could provide useful information for successful genotype selection and breeding. Therefore, these agronomically significant features were evaluated in ten black cumin genotypes (Afghanistan, Pakistan, Syria, India, Arak, Isfahan, Semirom, Shahreza, Shahrekord, and Mashhad) under three irrigation regimes (40% (I_1), 60% (I_2), and 80% (I_3) of permissible moisture discharge) during the 2017 to 2018 growing seasons. Water stress was shown to increase the levels of carotenoids (Cars), proline, total soluble carbohydrates (TSC), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), catalase (CAT), and ascorbate peroxidase (APX) activities but reduced the relative water content (RWC) and chlorophyll content. The highest increases in Cars, TSC, proline, CAT, and APX were noted in the Arak, Isfahan, Semirom, Shahreza, Shahrekord, and Mashhad genotypes under the I_3 water regime, respectively. At the same time, the lowest decrease was observed in chlorophyll, H_2O_2, and relative water content (RWC) in Semirom. According to the stress susceptibility index, the most resistant genotypes were Shahrekord under I_2 and Semirom under I_3. These data demonstrate that the irrigation regimes affected the physiological, biochemical, and morphological features of black cumin both qualitatively and quantitatively, although the impact varied depending upon the genotype, irrigation regime, and traits. As such, the results presented represent valuable information with which to inform future selection and breeding programs for drought-tolerant black cumin. This is of particular significance considering global climate change.

Keywords: medicinal plant; black cumin; irrigation regime; chlorophyll; proline; principal component analysis

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1. Introduction

Drought is widely acknowledged as one of the most limiting abiotic stresses affecting global agricultural productivity [1–6]. Climate change, insufficient and variable distribution of rainfall, population increase, and agricultural mismanagement have all contributed to water shortages, which have harmed the growth and development of crop species [2,7–12]. The effect of drought stress on plants is influenced by the level of drought and its duration, environmental factors, such as salt and heat stress, the genotype of the plant, and the plants' life cycle [13–16]. The adverse effects of drought can be mitigated by implementing a variety of innovative management strategies. Under drought stress, some aromatic and medicinal plant species develop secondary metabolites with significant economic value [4]. Zali and Ehsanzadeh found that drought stress increased the production of

leaf polyphenol and essential oil concentrations in fennel (*Foeniculum vulgare* Mill.) [7,17]. Several studies have also shown that drought had beneficial impacts on secondary metabolite biosynthesis, solute accumulation, and enzyme activity [5,8–10,18–22]. Cultivation of drought-tolerant genotypes of medicinal and aromatic plant species, on the other hand, could be another way to address water scarcity issues. The success of this approach was reported in some crops, such as caraway [22], pot marigold [23], cumin [24], and several other species [25]. Drought-resistant fennel genotypes, such as Shiraz and Yazd, demonstrated an increase in the accumulation of osmotic solutes and carotenoids, according to Askari and colleagues [26,27]. It is also feasible to boost bioactive plant components by inducing the formation of secondary metabolites under the most appropriate irrigation regime. For example, higher levels of secondary metabolites in pot marigold were obtained under 60 percent depletion of available soil water [23]. Alinian and colleagues suggested that an irrigation regime of 150 and 200 mm evaporation (based on a Class A pan) in cumin could result in higher leaf flavonoid and seed phenolic contents, respectively [24]. Laribi and collaborators also reported on the selection of appropriate irrigation regimes to increase the secondary metabolite production in caraway [22]. In this regard, other authors reported good results in chamomile (*Matricaria chamomilla*) [28] and in *Thymus carmanicus* [29]. The literature demonstrates that, while medicinal plant yields may be reduced, considerable gains in secondary metabolite production can be achieved by adopting drought-tolerant species and genotypes within species in conjunction with an appropriate watering scheme.

Black cumin (*Nigella sativa* L.) is a medicinal plant in the Ranunculaceae family, mostly grown for its seed and oil production. The traditional medicine, pharmaceutical, and food industries commonly use black cumin [30,31]. Anthelmintic, antiviral, antibacterial, antipyretic, galactagogue, carminative, and blood sugar-reducing properties are all ascribed to the seeds of this species [31]. The seed yield of black cumin was reduced by water stress, but the essential oil, thymoquinone, and carvone contents were concomitantly increased [32]. However, further, more detailed investigation of the physiological, biochemical, and morphological response of black cumin genotypes to different irrigation regimes is required. Thus, the aim of the current study is to investigate how a selection of widely cultivated black cumin genotypes respond to different watering regimes and degrees of drought stress.

2. Materials and Methods

2.1. Experimental Arrangement

To evaluate the growth performance of black cumin, an open field experiment was performed at the College of Agriculture, Isfahan University of Technology Experimental Station located in Levark, Nejaf-Abad (latitude: 32° 32 N and longitude: 51 23° E; altitude: 1630 m) Isfahan, Iran during the 2017 and 2018 growing seasons. The soil consisted of fine loam with a pH of 7.8, an EC of 1.8 dS m^{-1}, a bulk density of 1.47 g cm^{-3}, a soil organic C content of 3.8 g kg^{-1}, and nitrogen, phosphorus, and potassium levels of 420, 23.6, and 250 mg kg^{-1}, respectively. The geographical region has an average annual rainfall of 140 mm and an average annual temperature of 14 °C. The ten selected genotypes of black cumin (Arak, Isfahan, Semirom, Shahreza, ShahrKord, Mashhad, Afghanistan, Pakistan, India, and Syria) were subjected to three irrigation regimes: 40% (I_1), 60% (I_2), and 80% (I_3) of permissible humidity discharge from soil, indicating normal, moderate, and severe stress, respectively. In March 2017 and 2018, the black cumin seeds were planted in rows, spaced 5 cm apart. Each subplot consisted of four rows, 25 cm apart and 3 m in length. Drought stress was initiated when the plants reached the fourth stage of leaf development.

2.2. Irrigation Treatment

According to the assessment of water content in the root growth zone before irrigation, three degrees of irrigation (40% (I_1), 60% (I_2), and 80% (I_3)) of permitted moisture discharge from soil were performed. The percentage increase or decrease of the different parameters was calculated considering I_1 as the control group. Two soil samples from each subplot

were taken at depths of 30 cm, 72 h after irrigation, to measure the moisture content in order to determine the water capacity of the soil profile under the three irrigation treatments. Equation (1) was used to compute the volume of water for each plot. The irrigation time was determined using Equations (2)–(4).

$$V_W = \frac{ASW \times P}{E_a} \quad (1)$$

In Equation (1), V_W was the volume of water per irrigation in m^3, E_a denotes the irrigation water use efficiency, and P denotes the maximum rate at which soil moisture can be discharged (40% control, medium stress 60%, and severe stress 80%).

$$ASW = (\theta_{FC} - \theta_{PWP}) \times P_b \times V \quad (2)$$

In Equation (2), ASW represents available soil moisture, FC represents the percentage of soil water content in the crop capacity point, PWP represents the percentage of moisture content at the permanent wilting point, P_b represents soil bulk density at the root growth zone in grams per cm^3, and V represents the experimental unit size.

$$V = (A \times D) \quad (3)$$

In Equation (3), A is the square meter area of the main plot (length × breadth of the experimental plot), and D is the depth of root development.

$$\theta_{irrig} = \theta_{FC} - (\theta_{FC} - \theta_{PWP}) \times P \quad (4)$$

In Equation (4), θ_{irrig} = volumetric soil moisture content.

2.3. Relative Water Content (RWC) and Photosynthetic Pigments Measurements

Chlorophyll content and *RWC* were measured at the 50% blooming stage of the crop. The second fully grown top leaves were used to calculate *RWC*. The weight of fresh leaves was measured immediately after they were excised. The turgid mass was calculated after the excised leaves were placed in distilled water for 4 h at 23 °C. After drying at 70 °C for 48 h, the dry biomass was measured. Finally, using Equation (5) [33], *RWC* was calculated:

$$RWC\% = \left(\frac{Fresh\ Weight - Dry\ Weight}{Turgid\ Weight - Dry\ Weight} \right) \times 100 \quad (5)$$

Fresh leaf material (200 mg) was obtained from completely expanded leaves of each treatment to measure the chlorophyll concentration. Samples were stored at −30 °C until the next step. The frozen leaves were crushed and extracted with 10 mL of acetone 80% (v/v). The slurry was filtered, centrifuged at 5000× g for 10 min (5810R, Eppendorf Refrigerated Centrifuge, Germany), and Chlorophyll a (Chl-a), Chlorophyll b (Chl-b), and Carotenoids (Cars) concentrations were measured using a spectrophotometer (U-1800 UV/VIS, Hitachi, Japan) at 645, 663, and 470 nm, respectively. Acetone (80% v/v) was used as the blank. According to Lichtenthaler and Wellburn, Chl-a, Chl-b, and Cars concentrations were determined and reported as $mg \cdot g^{-1}$ leaf fresh weight.

2.4. Determination of MDA, H_2O_2, Proline, and Total Soluble Carbohydrates Concentrations

These traits were measured at the 50% blooming stage of the crop. Malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) reaction, and the level of lipid peroxidation was quantified in terms of thiobarbituric acid reaction substances (TBARS) concentration according to previously reported protocols [34]. Fresh leaf material (100 mg) was frozen and stored at −30 °C until the analyses were performed. To homogenize the leaf samples, 0.5 mL of 0.1% TCA was used, followed by centrifugation at 10,000× g for 5 min. After centrifugation, 4 mL of 0.5% TBA in 20% TCA was added to 1 mL of the supernatant

and heated for 30 min to 95 °C before quick cooling in an ice bath. The suspended turbidity was removed by centrifugation at 10,000× *g* for 15 min. The absorbance of the resultant supernatant was measured at 532 nm using a spectrophotometer (U-1800 UV/VIS, Hitachi, Japan). Nonspecific absorption was acquired at 600 nm and deducted. The TBARS concentration was determined using a 150 mM^{-1} cm^{-1} absorption coefficient, and the results were reported in mmol MDA g^{-1} leaf fresh mass.

The Bates method was used to determine proline content [35]. In particular, 500 mg of fully formed leaves from the plant's apex was homogenized in a 3% aqueous solution of sulfosalicylic acid (10 mL), followed by filtration of the resulting product. Then, 2 mL of glacial acetic acid, 2 mL of ninhydrin reagents, and 2 mL of filtered extract were added. The mixture was boiled for 1 h at 100 °C before being cooled on ice. The toluene phase was separated and its absorbance measured at 520 nm after thoroughly mixing 4 mL of toluene with the cooled mixture.

Dried leaf material (0.1 g) was crushed, mixed in 13 mL of 80% ethanol, and centrifuged for 10 min to determine total soluble carbohydrate (TSC) content in the samples. The homogenized extract was then mixed with 1 mL of phenol 5% and 5 mL of sulfuric acid. After 30 min, the absorbance of the reaction extract was determined spectrophotometrically at 490 nm [36].

The H_2O_2 concentration was measured as described by Velikova and colleagues [37]. Leaf material (200 mg) was homogenized in 2 mL 0.1% (*w*/*v*) TCA and centrifuged at 10,000× *g* at 4 °C for 10 min, and 0.5 mL of the supernatant was added to 0.5 mL potassium-phosphate buffer (0.1M, pH 7.0) and 1 mL of KI (1M). The blend was incubated at room temperature for 1 h, then processed by measuring the retention at 390 nm. The H_2O_2 concentration was calculated using a standard curve.

2.5. Antioxidant Enzyme Activity

Catalase (CAT) and ascorbate peroxidase (APX) activities were measured by using fresh leaf material (0.1 g). The leaf samples were ground to a fine powder in a pre-chilled mortar and pestle using liquid nitrogen. Once the samples had been powdered, the powder was added to 1 mL of 50 mM sodium-phosphate buffer (pH 7) containing 2 mM dithiothreitol, 2 mM EDTA, 0.2% Triton X-100, and 50 mM Tris–HCl. The resultant sample was centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was used for measurement of CAT and APX activities [38]. Soluble protein was determined using bovine serum albumin (BSA) at 595 nm by a UV/VIS spectrophotometer according to the method described by Bradford [39].

Catalase activity was measured in a total volume of 3 mL of 50 mM sodium-phosphate buffer (pH 7.0) containing 4.51 µL of H_2O_2 (30%) and 50 µL of enzyme extract (pH 7.8). H_2O_2 diminishment was measured at 240 nm every 30 s for 2 min using a spectrophotometer (U-1800, Hitachi, Japan). The sum of each catalase reaction was calculated using the following equation:

$$(U)CAT\ activity = \frac{\Delta A \times TV \times D}{\varepsilon \times EV}$$

$$\left(\frac{U}{mL}\right)CAT\ volumetric\ activity = \frac{CAT\ activity}{Unite\ volume}$$

$$\left(\frac{U}{mg\ Protein}\right)Specific\ CAT\ activity = \frac{CAT\ volumetric\ activity\left(\frac{U}{mL}\right)}{Extract\ protein\ concentration\left(\frac{mg}{mL}\right)}$$

U = A unit of catalase activity is equal to the amount of enzyme that catalyzes the H_2O_2 to O_2 and H_2O in one min.

ΔA = Differences in absorbance in 240 nm in one min

TV = Total bulk of buffer and extract (3 mL)

EV = Extract bulk (0.05 mL)

ε = Extinction coefficient for catalase (39.4 $mM^{-1}cm^{-1}$)

D = Dilution factor

Ascorbate peroxide activity was measured in a total volume of 3 mL of 50 mM sodium-phosphate buffer (pH 7.0) containing 4.51 µL of H_2O_2 (30%), 100 µL of 5 mM ascorbate, and 50 µL of enzyme extract (pH 7.8). Ascorbate peroxide activity was measured at 290 nm every 30 s for 2 min using a spectrophotometer (U-1800, Hitachi, Japan). The sum of particular ascorbate peroxide activity was calculated utilizing the same equation utilized for particular catalase activity, whereas $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$.

2.6. Stress Susceptibility Index

The Fischer and Maurer method [40] was used to calculate the stress susceptibility index (*SSI*) using the following equation:

$$SSI = \left(1 - \frac{Y_{si}}{Y_{ni}}\right) / SI \quad (6)$$

The stress intensity (*SI*) is a number that varies from zero to one, and it is calculated using the following equation.

$$SI = 1 - \left(\frac{Y_S}{Y_N}\right) \quad (7)$$

Drought tolerance is indicated by a decreased index of stress susceptibility. Y_{si} denotes genotype performance (yield) in stressful environments, Y_{ni} signifies genotype grown in non-stressful situations, Y_S represents the average of genotypes grown in stressful conditions, and Y_N denotes the mean of genotypes produced in non-stressful settings in the above equations.

2.7. Statistical Analysis

The least significant difference (LSD, $p < 0.05$) in SAS (ver. 9.4) software was used to compare the means of the physiological, morphological, and biochemical variables. SAS was also used to perform principal component analysis (PCA) and cluster analysis, while Stat Graphics was used to compose graphical representations of the data.

3. Results and Discussion

In our initial investigation, the effect of the different growing seasons (2017–2018) was evaluated. No significant differences were found in any of the traits except for total soluble carbohydrate contents and the ratio of Chl-a and Chl-b concentrations. Therefore, the data for the two years were combined with the different irrigation regimes (I × Y), and no differences were observed (Table 1). However, when the interaction effects between irrigation regime × genotype were evaluated, significant results were observed for all the measured traits except for the ratio of Chl-a and Chl-b concentrations (Table 1).

Table 1. Analyses of variance for different traits of ten black cumin genotypes (G) at three irrigation regimes (I) in two years (Y).

Sources of Changes	DF	Chl-a	Chl-b	Chl-a/b	Chl-a+b	Cars	Proline	TSC	RWC	H_2O_2	MDA	CAT	APX
I	2	0.3050 **	0.2527 **	9.053 **	1.1108 **	0.0243 **	27.10 **	12.534 **	1114.42 **	2.117 **	1377.4 **	0.1271 **	4.67 **
Y	1	0.0081 ns	0.0028 ns	0.0144 *	0.0207 ns	0.000004 ns	0.011 ns	0.524 *	8.97 ns	0.0061 ns	2.52 ns	0.0005 ns	0.015 ns
I × Y	2	0.0023 ns	0.0014 ns	0.1309 ns	0.0012 ns	0.00003 ns	0.004 ns	0.201 ns	3.59 ns	0.001 ns	0.2290 ns	0.0020 ns	0.061 ns
Error	12	0.0022	0.0022	0.2613	0.0050	0.0001	0.4376	0.143	9.50	0.003	2.21	0.0015	0.055
G	9	0.0993 **	0.0095 **	1.1318 **	0.1550 **	0.0034 **	8.52 **	6.098 **	125.77 **	0.168 **	302.18 **	0.0860 **	1.307 **

Table 1. *Cont.*

Sources of Changes	DF	Chl-a	Chl-b	Chl-a/b	Chl-a+b	Cars	Proline	TSC	RWC	H_2O_2	MDA	CAT	APX
G × Y	9	0.0205 ns	0.0017 ns	0.7795 ns	0.0021 ns	0.0001 **	0.0565 ns	0.096 ns	13.53 ns	0.0008 ns	7.52 ns	0.0018 ns	0.029 ns
G × I	18	0.0087 **	0.0033 **	0.3848 ns	0.0185 **	0.0002 **	0.5589 **	0.350 **	17.39 **	0.015 **	8.927 **	0.0061 **	0.144 **
I × Y × G	18	0.0026 ns	0.0006 ns	0.1745 ns	0.0038 ns	0.00003 ns	0.0715 ns	0.114 ns	9.81 ns	0.001 ns	6.545 ns	0.0026 **	0.053 ns
Error	108	0.0043	0.0020	0.0057	0.3529	0.00006	0.231	0.124	6.67	0.007	4.330	0.0009	0.062

DF, degrees of freedom; Chl-a, chlorophyll a concentration; Chl-b, chlorophyll b concentration; ratio of chlorophyll a and chlorophyll b concentrations; Cars, leaf carotenoids concentration; TSC, total soluble carbohydrates; RWC, relative water content; H_2O_2—hydrogen peroxide; MDA—malondialdehyde; CAT—catalase; APX—ascorbate peroxidase. ^{ns}, non-significant. * Significant at $p \leq 0.05$. ** Significant at $p \leq 0.01$.

3.1. Relative Water Content (RWC)

The relative water content was found to reduce as the water stress level increased, and the observed reduction in RWC was specific for both the drought level and plant genotype. The RWC ranged from 70% in Pakistan to 75% in Arak under I_1 (Table 2). The highest RWC was observed in Shahrekord (72%) under I_2 and in Semirom (68%) under I_3. The lowest RWC was recorded in the Pakistan genotype: both I_2 (62%) and I_3 (54%) (Table 2). These results are in agreement with those previously recorded by Zali and Ehsanzadeh [17] and Askari and Ehsanzadeh in fennel [26,27], and Maghsoodi and colleagues in alfalfa [41], who reported that a water deficit led to a decrease in RWC and the extent of the decrease was genotype-specific.

Table 2. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on Cars and RWC of black cumin.

	Cars (mg.g^{-1}FW)				RWC (%)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	0.23 $^{k\,\dagger}$	0.26 ^{gh} (+15) ‡	0.26 ^{hi} (+12)	0.256 ^{F}	71.53 $^{a–d}$	68.66 $^{a–e}$ (−5)	64.81 $^{d–f}$ (−10)	68.33 ^{AB}
Pakistan	0.23 ^{k}	0.26 ^{gh} (+15)	0.26 ^{hi} (+13)	0.256 ^{F}	70.60 $^{a–d}$	62.00 $^{e–g}$ (−12)	54.84 ^{g} (−24)	62.48 ^{C}
Syria	0.23 ^{k}	0.26 $^{g–i}$ (+14)	0.26 ^{i} (+12)	0.255 ^{F}	71.17 $^{a–d}$	65.14 $^{c–e}$ (−9)	59.78 ^{fg} (−16)	65.37 ^{BC}
India	0.26 ^{hi}	0.28 ^{ef} (+15)	0.27 ^{fg} (+5)	0.266 ^{DE}	73.02 $^{a–c}$	67.49 $^{a–e}$ (−8)	65.03 $^{b–g}$ (−10)	68.49 ^{AB}
Arak	0.26 ^{hi}	0.30 ^{b} (+16)	0.29 ^{cd} (+12)	0.286 ^{B}	75.40 ^{a}	68.23 $^{a–e}$ (−10)	67.46 $^{a–e}$ (−11)	70.36 ^{A}
Isfahan	0.23 ^{k}	0.26 $^{g–i}$ (+14)	0.26 ^{hi} (+12)	0.256 ^{F}	73.29 ^{ab}	68.43 $^{a–e}$ (−7)	65.10 $^{b–g}$ (−12)	68.94 ^{AB}
Semirom	0.24 ^{jk}	0.27 ^{fg} (+14)	0.26 $^{g–i}$ (+11)	0.261 ^{EF}	73.30 $^{a–c}$	70.66 $^{a–d}$ (−4)	68.28 $^{a–e}$ (−7)	70.74 ^{A}
Shahreza	0.24 ^{j}	0.28 ^{e} (+14)	0.30 ^{bc} (+22)	0.277 ^{C}	72.96 $^{a–c}$	68.60 $^{a–e}$ (−7)	62.19 $^{l–n}$ (−15)	68.91 ^{AB}
Shahrekord	0.24 ^{j}	0.27 ^{ef} (+13)	0.29 ^{d} (+20)	0.272 ^{CD}	74.02 ^{ab}	72.48 $^{a–d}$ (−3)	67.48 $^{a–f}$ (−9)	71.24 ^{A}
Mashhad	0.26 ^{hi}	0.30 ^{bc} (+14)	0.31 ^{a} (+18)	0.294 ^{A}	74.75 ^{ab}	67.48 $^{a–f}$ (−10)	65.37 $^{b–g}$ (−13)	69.20 ^{A}
Avg.	0.245 ^{B}	0.279 ^{A}	0.280 ^{A}		73.02 ^{A}	67.89 ^{B}	64.33 ^{C}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase or uppercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1).

Under water stress, the plant water content plays a significant function in modulating or activating the antioxidant defense mechanism [13,14]. The RWC refers to water uptake by the roots and water loss by transpiration. It is used as a reliable indicator of water content compared to the maximum water content in plants and is indicative of the plant hydration level [42].

3.2. Chlorophyll-a, Chlorophyll-b, and Carotenoid Content

An interaction between the genotype and irrigation regime on the Chl-a, Chl-a+b, and Cars concentrations was observed at the 1% probability level (Tables 2 and 3).

Table 3. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on Chl-a and Chl-a+b of black cumin.

	Chl-a (mg g^{-1} FW)				Chl-a+b (mg g^{-1}FW)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	0.410 $^{mn\ \dagger}$	0.479 $^{k–l}$ (−14) ‡	0.384 ^{n} (−19)	0.424 ^{C}	0.683 $^{i–n\ \dagger}$	0.707 $^{j–i}$ (−4) ‡	0.554 ^{p} (−11)	0.648 ^{C}
Pakistan	0.545 $^{d–h}$	0.449 ^{lm} (−17)	0.411 ^{mn} (−25)	0.668 ^{C}	0.796 $^{f–i}$	0.660 $^{l–n}$ (−17)	0.590 $^{n–p}$ (−25)	0.682 ^{C}
Syria	0.508 $^{i–l}$	0.399 ^{mn} (−21)	0.374 ^{n} (−26)	0.427 ^{C}	0.786 $^{g–i}$	0.624 $^{m–o}$ (−20)	0.530 ^{p} (−32)	0.647 ^{C}
India	0.662 $^{b–d}$	0.537 $^{g–k}$ (−18)	0.497 $^{j–l}$ (−24)	0.553 ^{B}	0.918 ^{de}	0.769 $^{h–j}$ (−16)	664 $^{l–n}$ (−28)	0.784 ^{B}
Arak	0.739 ^{a}	0.584 $^{d–g}$ (−20)	0.505 $^{i–l}$ (−31)	0.610 ^{AB}	1.093 ^{ab}	0.841 $^{f–g}$ (−23)	0.649 $^{l–n}$ (−40)	0.861 ^{A}
Isfahan	0.641 $^{b–d}$	0.550 $^{e–j}$ (−14)	0.522 $^{h–k}$ (−18)	0.571 ^{AB}	0.952 ^{cd}	0.799 $^{f–i}$ (−16)	0.708 $^{j–l}$ (−25)	0.820 ^{AB}
Semirom	0.611 $^{c–e}$	0.581 $^{d–h}$ (−5)	0.533 $^{j–k}$ (−13)	0.575 ^{AB}	0.955 ^{cd}	0.866 ^{ef} (−9)	0.762 ^{ij} (−20)	0.861 ^{A}
Shahreza	0.673 ^{bc}	0.562 $^{e–i}$ (−17)	0.509 $^{i–l}$ (−24)	0.581 ^{AB}	0.973 ^{cd}	0.811 $^{f–i}$ (−17)	0.710 $^{j–l}$ (−28)	0.277 ^{C}
Shahrekord	0.683 ^{ab}	0.592 $^{d–g}$ (−13)	0.520 $^{h–k}$ (−24)	0.598 ^{AB}	1.020 ^{bc}	0.848 $^{e–g}$ (−16)	0.742 $^{j–k}$ (−27)	0.870 ^{A}
Mashhad	0.742 ^{a}	0.606 $^{d–f}$ (−18)	0.504 $^{i–l}$ (−32)	0.618^{A}	1.100 ^{a}	0.852 $^{e–g}$ (−18)	0.651 $^{l–n}$ (−32)	0.868 ^{A}
Avg.	0.618 ^{A}	0.534 ^{B}	0.476 ^{C}		0.928 ^{A}	0.787 ^{B}	0.656 ^{C}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase or uppercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1).

Drought stress caused a decrease in Chl-a, Chl-b, and Chl-a+b but increased the Chl-a/b ratio and Cars concentrations (Table 2). Under the I_2 and I_3 irrigation regimes, Chl-a decreased by 14 and 23%, Chl-b decreased by 17 and 33%, Chl-a+b declined by 16 and 28%, while Chl-a/b increased by 11 and 37%, respectively (Table 4).

Table 4. Mean comparison of the effect of irrigation regime (I) and genotype (G) on Chl-a, Chl-a, Chl-a/b, Chl-a+b, and Cars of black cumin in a two-year period (2017–2018).

	Chl-a (mg g^{-1} FW)	**Chl-b (mg g^{-1} FW)**	**Chl-a/b (mg g^{-1} FW)**	**Chl-a+b (mg g^{-1} FW)**	**Cars (mg g^{-1} FW)**
Irrigation regime (I)					
I_1	0.618 ^{a}	0.309 ^{a}	2.003 ^{b}	0.928 ^{a}	0.245 ^{b}
I_2	0.534 ^{b} (−14)	0.243 ^{b} (−17)	2.260 ^{a} (+11)	0.778 ^{b} (−16)	0.279 ^{a} (+14)
I_3	0.476 ^{c} (−23)	0.181 ^{c} (−33)	2.792 ^{a} (+37)	0.656 ^{b} (−28	0.280 ^{a} (+14)
Genotypes					
Afghanistan	0.424 ^{c}	0.232 $^{c–e}$	2.046 ^{c}	0.648 ^{c}	0.256 ^{f}
Pakistan	0.468 ^{b}	0.214 ^{e}	2.253 $^{a–c}$	0.682 ^{c}	0.256 ^{f}
Syria	0.427 ^{c}	0.219 ^{de}	2.081 ^{bc}	0.647 ^{c}	0.255 ^{f}
India	0.553 ^{b}	0.230 $^{c–e}$	2.549 ^{ab}	0.784 ^{b}	0.266 ^{de}
Arak	0.610 ^{ab}	0.251 ^{bc}	2.490 ^{c}	0.861 ^{a}	0.286 ^{b}
Isfahan	0.571 ^{ab}	0.248 $^{b–d}$	2.315 $^{a–c}$	0.820 ^{ab}	0.256 ^{cd}
Semirom	0.575 ^{ab}	0.286 ^{a}	2.088 ^{bc}	0.861 ^{a}	0.261 ^{ef}
Shahreza	0.581 ^{ab}	0.250 $^{b–d}$	2.484 $^{a–c}$	0.832 ^{ab}	0.277 ^{c}
Shahrekord	0.598 ^{ab}	0.271 ^{ab}	2.260 $^{a–c}$	0.870 ^{a}	0.272 ^{cd}
Mashhad	0.618 ^{a}	0.249 $^{b–d}$	2.738 ^{a}	0.868 ^{a}	0.294 ^{a}

In each column and within each experimental factor, means with at least one similar letter are not significantly different according to the LSD ($p < 0.05$).

The highest decrease in the Chl-a concentration (−32%) was found in the Mashhad genotype under the I_3, while the lowest (−5%) was obtained under I_2 in Semirom (Table 3). Our results highlighted that the Chl-a and Chl-b concentrations decreased following drought stress, as previously reported [24]. Furthermore, an increase in the Chl-a/b

ratio was recorded, probably due to the degradation of Chl-b into Chl-a during drought, as observed by several authors [26,27,43].

Drought stress may also cause an increase in Chl breakdown and inhibition of the production of photosynthetic pigments, thereby decreasing leaf Chl concentrations [44]. Furthermore, the loss or decline in Chl concentration may be due to the loss of chloroplast membranes, lamellae dispersion, or excessive edema [45]. A decrease in the Chl content during drought stress is a common marker of oxidative stress, which could be caused by pigment photo-oxidation and chlorophyll degradation. Chlorophyll and other photosynthetic pigments are essential for the light-harvesting process and lowering power output [46]. Higher Chl content and stability have been reported to be associated with drought tolerance [45].

With regard to carotenoids, a significant and growing increase was observed in all the experimental groups (I_2 and I_3) subjected to water stress compared to the control (I_1). In particular, the plants subjected to water stress I_2 showed a fairly uniform increase (13–16%). Greater water stress resulted in more heterogeneous responses. Indeed, Shahreza, Shahrekord, and Mashhad showed an increase of 22, 20, and 18%, respectively, and India only 5%. The other plants maintained an increase in the carotenoid content of between 11 and 13%. Moreover, the genotype 'Pakistan' was shown to produce the smallest Cars concentrations, and Mashhad had the greatest under I_1 (Table 2). In sesame and in alfalfa under drought stress, the Cars concentrations increased in some genotypes and decreased in others [29,41]. Other authors reported similar increases in Cars content during drought stress in *Zea mays* and in *Calendula officinalis* [47,48]. In conditions of abiotic stress, such as drought, plants implement protective defenses, such as increasing the content of carotenoids [49]. Carotenoids improve the plant's response to oxidative stress. In addition, the decrease in chlorophyll makes the carotenoid content percentage higher [49]. Our data indicate that the Shahreza genotype was drought-tolerant due to the highest increases in Cars content.

When plants are stressed by drought, Cars play an important role in signaling, neutralizing oxidative stress, and induction of abscisic acid production (an essential carotenoid-derived plant hormone that causes stomatal closure, thereby limiting transpiration) [50]. Under I_2 and I_3, Cars concentrations were shown to increase compared to the control (I_1) (Table 4).

3.3. Leaf Proline and Total Soluble Carbohydrates (TSC)

The highest proline content was detected in the Shahrekord genotype under I_1, I_2, and I_3, while the lowest was noted in the Afghanistan genotype under I_1, I_2, and I_3 (Table 5).

The highest increase in proline content was observed in the Syria genotype (+74%) under I_2 and in Arak (+95%) under the I_3, while the lowest was obtained under I_2 (+8%) and I_3 (+22%) in Semirom (Table 5). Under drought stress, the proline content was previously reported to increase in canola [51] and remained unchanged in the early stages of development, but it increased upon flowering in soybean [52]. Changes in proline content may be influenced by irrigation regimes, plant growth stages, environmental variables, and plant genotypes. A well-known eco-physiological process that allows plants to maintain a lower osmotic potential and withstand drought is the accumulation of solutes, such as proline and sugars, in the cytoplasm [53]. Kaur and Asthir showed that proline accumulated under water stress decreased the degradation and hydrolysis of protein [54]. Under drought stress, proline production and accumulation are known to be due to variations in the enzyme activities involved in proline biosynthesis, degradation, and oxidation inhibition [54].

Table 5. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on TSC and proline of black cumin.

	TSC (mg/FW)				Proline (µmol/g)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	1.51 $^{n–p}$ †	1.80 $^{l–n}$ (+20) ‡	2.66 $^{c–f}$ (+70)	1.99 ^{D}	1.78 ^{n} †	2.09 $^{m–n}$ (+18)	2.41 $^{l–n}$ (+36)	2.10 ^{E}
Pakistan	1.17 ^{p}	1.36 ^{op} (+16)	1.48 $^{n–p}$ (+25)	1.33 ^{E}	2.51 $^{l–m}$	3.63 $^{g–k}$ (+45)	4.44 $^{b–d}$ (+76)	3.53 ^{C}
Syria	1.16 ^{p}	1.37 ^{op} (+18)	1.65 $^{m–o}$ (+42)	1.39 ^{E}	2.02 $^{m–n}$	3.54 $^{h–k}$ (+74)	3.73 $^{f–j}$ (+85)	3.15 ^{D}
India	1.93 $^{j–m}$	2.54 $^{e–h}$ (+31)	2.64 $^{c–h}$ (+37)	2.37 ^{C}	2.54 $^{l–m}$	3.53 $^{h–k}$ (+39)	3.86 $^{d–g}$ (+52)	3.31 ^{CD}
Arak	2.21 $^{h–k}$	2.73 $^{c–f}$ (+24)	3.33 ^{b} (+50)	2.76 ^{B}	2.59 $^{l–m}$	3.83 $^{e–g}$ (+48)	4.99 $^{c–i}$ (+95)	3.50 ^{C}
Isfahan	1.36 ^{op}	1.59 $^{m–o}$ (+14)	1.75 $^{m–o}$ (+28)	1.56 ^{E}	2.50 $^{l–m}$	3.35 $^{j–k}$ (+34)	3.41 $^{i–k}$ (+37)	3.09 ^{D}
Semirom	2.14 $^{i–l}$	2.79 $^{c–e}$ (+31)	3.07 ^{bc} (+44)	2.67 ^{B}	3.69 $^{f–k}$	3.97 $^{d–j}$ (+8)	4.51 $^{f–i}$ (+22)	4.06 ^{B}
Shahreza	1.82 $^{k–n}$	2.33 $^{f–j}$ (+28)	2.65 $^{c–g}$ (+45)	2.27 ^{C}	3.02 $^{k–l}$	3.50 $^{h–k}$ (+16)	4.30 $^{b–g}$ (+43)	3.61 ^{C}
Shahrekord	2.40 $^{e–i}$	2.97 $^{b–d}$ (+24)	4.22 ^{a} (+75)	3.19 ^{A}	4.15 $^{c–h}$	4.65 $^{a–c}$ (+12)	5.26 ^{a} (+26)	4.69 ^{A}
Mashhad	2.26 $^{g–j}$	2.56 $^{d–h}$ (+14)	3.07 ^{bc} (+35)	2.63 ^{B}	2.66 $^{l–m}$	4.34 $^{b–d}$ (+64)	4.96 ^{ab} (+86)	3.98 ^{B}
Avg.	1.79 ^{C}	2.21 ^{B}	2.65 ^{A}		2.54	3.5	4.05	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase or uppercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1).

The highest TSC content was recorded in Shahrekord, under all three irrigation regimes, and the lowest was noted in Pakistan and Syria under I_1 and I_2, and in Pakistan under I_3 (Table 5). TSC increased from +14% in Mashhad and Isfahan to +31% in Semirom and India under I_2 and from +25% in Pakistan to +75% in Shehrekord under I_3 (Table 5).

The greatest TSC was noted in Shehrekord under I_1, while the lowest was obtained under I_3 in the Pakistan and Syria genotypes (Table 5). Others have also reported increases in the TSC levels in plants under water stress [17,23,26,27,54]. Askari and colleagues reported that drought-tolerant genotypes ('Yazd' and 'Kerman') produced higher total leaf soluble carbohydrates [26,27].

Soluble sugars are carbon and energy sources in cells, and some also play a role as signaling molecules in stress tolerance in plants via osmotic adjustment [55]. They may also feed the oxidative pentose pathway, which can result in ROS scavenging. As a result, variations in sugar concentration in conjunction with changes in the environment often impact ROS production, making sugars one of the most critical 'players' in the redox balance in plants [56]. In addition, plants can regulate their osmotic potential to increase the accumulation of soluble sugars, which improves the plant's water holding capacity and reduces water stress [57]. Our TSC results indicate that Shahrekord, under all irrigation regimes, is the preferred genotype for drought tolerance.

3.4. Antioxidant Enzyme Activities

Under drought, catalase (CAT) and ascorbate peroxidase (APX) activities increased, although the increase was drought level- and genotype-specific (Table 6). In the current study, the CAT activity was the highest in Arak under I_1 and in Semirom under the I_2 and I_3 irrigation regimes, while it was the lowest in Afghanistan and Syria under I_1 and in Isfahan and Syria under the I_2 and I_3 regimes, respectively (Table 6). Additionally, the highest increases in CAT activities were noted in Semirom (+60%) under I_2 and in Shehrekor (+81%) under I_3 treatments. However, the lowest increases in CAT activities in India were marked under I_2 (+9%) and I_3 (+16%), respectively (Table 6).

Table 6. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on CAT and APX of black cumin.

	CAT (Unit mg^{-1} Protein)				APX (Unit mg^{-1} Protein)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	0.143 ^{p} †	0.229 $^{i–o}$ (+58) ‡	0.217 $^{j–o}$ (+52)	0.196 ^{EF}§	1.23 $^{k–n}$ †	1.50 $^{h–k}$ (+21)	1.64 $^{g–i}$ (+33)	1.46 $^{C–E}$
Pakistan	0.203 $^{k–p}$	0.256 $^{f–l}$ (+26)	0.282 $^{e–i}$ (+38)	0.247 ^{CD}	1.21 ^{mn}	1.47 $^{i–n}$ (+21)	1.57 $^{g–j}$ (+28)	1.42 ^{DE}
Syria	0.145 ^{p}	0.169 ^{op} (+16)	0.187 $^{m–p}$ (+28)	0.167 ^{F}	1.19 ^{n}	1.38 $^{i–n}$ (+14)	1.49 $^{i–l}$ (+25)	1.36 ^{E}
India	0.195 $^{l–p}$	0.213 $^{j–o}$ (+9)	0.226 $^{i–o}$ (+16)	0.211 ^{DE}	1.34 $^{j–n}$	1.60 $^{g–j}$ (+23)	1.79 $^{e–g}$ (+32)	1.57 $^{B–D}$
Arak	0.267 $^{f–j}$	0.299 $^{d–g}$ (+12)	0.334 ^{de} (+25)	0.300 ^{B}	1.33 $^{j–n}$	1.66 $^{f–i}$ (+23)	1.82 $^{d–g}$ (+35)	1.42 ^{DE}
Isfahan	0.151 ^{p}	0.173 ^{op} (+15)	0.180 $^{n–p}$ (+19)	0.168 ^{F}	1.22 $^{l–n}$	1.48 $^{i–m}$ (+21)	1.58 $^{g–j}$ (+30)	1.60 ^{BC}
Semirom	0.257 $^{f–k}$	0.408 ^{bc} (+60)	0.433 ^{ab} (+69)	0.366 ^{A}	1.54 $^{g–j}$	2.07 ^{cd} (+34)	2.77 ^{a} (+79)	2.13 ^{A}
Shahreza	0.214 $^{j–o}$	0.309 $^{d–f}$ (+44)	0.346 ^{d} (+62)	0.346 ^{B}	1.49 $^{i–m}$	1.78 $^{e–h}$ (+20)	1.94 $^{c–f}$ (+31)	1.73 ^{B}
Shahrekord	0.236 $^{h–n}$	0.347 ^{cd} (+47)	0.429 ^{a} (+81)	0.290 ^{B}	1.57 $^{g–j}$	2.17 ^{c} (+38)	2.74 ^{b} (+75)	2.07 ^{A}
Mashhad	0.216 $^{j–o}$	0.242 $^{g–m}$ (+15)	0.294 $^{d–g}$ (+37)	0.250 ^{C}	1.44 $^{i–n}$	1.65 $^{g–i}$ (+15)	2.03 $^{c–e}$ (+40)	1.70 ^{B}
Avg.	0.203 ^{B}	0.264 ^{A}	0.293 ^{A}		1.35 C	1.67 ^{B}	1.91 ^{A}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1). § The means indicated with the same capital letters in the rows (main effect of the genotype) and in the columns (main effect of the irrigation regime) for each trait are not significantly different at the 5% probability level.

Catalase scavenges the oxidant H_2O_2 by decomposition to oxygen and water [56]. Catalase activity was shown to increase in *Arabidopsis* drought-tolerant genotypes when exposed to water stress, suggesting that this could be an adaptive response to ROS [58]. In our study, APX activity also increased, and the most significant increase was obtained for Shahrekord under I_2 (+38%) and Semirom under I_3 (+79%). In contrast, the Syria genotype displayed the lowest increase under I_2 (+14%) and I_3 (+25%), respectively (Table 6). In plant cells, APX, a key enzyme in the ascorbate-glutathione cycle, plays a crucial part in the detoxification system [59]. In line with our results, Shigeoka et al. showed that APX activity was increased under drought stress, alongside other enzymes [60]. However, in contrast to our findings, Zali and Ehsanzadeh found that drought stress reduced peroxidase and ascorbate peroxidase activities in fennel by 16 and 20%, respectively [17]. Several previous studies have found that, under drought stress, the antioxidant enzyme activity increased in drought-tolerant genotypes, which is consistent with our findings [23,26,27,29].

3.5. Malondialdehyde and Hydrogen Peroxide (H_2O_2) Analysis

The highest increase in malondialdehyde (MDA) was recorded in Mashhad (+32%) and Syria (+32%) under I_2 and Syria (+58%) under I_3, while the lowest increase was noted in Shahreza (+14%) and Shahrekord (+16%) under I_2, and Isfahan (+31%) under I_3 (Table 7).

Table 7. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on H_2O_2 and MDA of black cumin.

	H_2O_2 (μmol·g^{-1} FW)				MDA (nmol·g^{-1} FW)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	0.3916 ^{lm} †	0.5526 $^{d–g}$ (+41) ‡	0.7148 ^{bc} (+82)	0.552 ^{CD} §	28.86 $^{d–j}$ †	35.67 ^{ac} (+24)	40.07 (+39)	34.87 ^{A}
Pakistan	0.4033 $^{h–m}$	0.5688 $^{d–f}$ (+40)	0.6871 ^{bc} (+70)	0.553 ^{CD}	26.03 $^{f–l}$	31.17 $^{b–g}$ (+20)	35.97 $^{a–c}$ (+38)	31.06 ^{B}
Syria	0.3536 ^{ml}	0.5405 $^{d–g}$ (+54)	0.6990 ^{bc} (+95)	0.531 ^{D}	23.04 $^{i–m}$	30.27 $^{b–h}$ (+32)	36.42 ^{ab} (+58)	29.91 ^{BC}
India	0.4516 $^{g–l}$	0.6420 ^{cd} (+42)	0.7036 ^{bc} (+55)	0.599 ^{BC}	23.27 $^{i–m}$	29.22 $^{d–i}$ (+26)	34.48 $^{a–d}$ (+48)	28.99 ^{BC}
Arak	0.4848 $^{f–j}$	0.6310 $^{c–e}$ (+32)	0.7886 ^{ab} (+62)	0.634 ^{B}	26.82 $^{d–l}$	32.76 $^{b–e}$ (+22)	37.95 ^{b} (+42)	32.01 ^{AB}
Isfahan	0.3488 ^{ml}	0.3783 $^{j–m}$ (+10)	0.5346 $^{e–g}$ (+55)	0.420 ^{E}	27.30 $^{e–l}$	34.99 $^{a–d}$ (+28)	35.60 $^{a–c}$ (+31)	32.63 ^{AB}

Table 7. *Cont.*

	H_2O_2 (μmol·g^{-1} FW)				MDA (nmol·g^{-1} FW)			
Semirom	0.3253 ^{m}	0.3543 $^{k–m}$ (+8)	0.4081 $^{h–m}$ (+25)	0.362 ^{F}	21.12 $^{k–m}$	26.02 $^{f–l}$ (+23)	29.83 $^{d–h}$ (+41)	25.65 ^{D}
Shahreza	0.3705 $^{k–m}$	0.4636 $^{f–k}$ (+27)	0.5351 $^{e–g}$ (+46)	0.456 ^{E}	21.89 $^{k–m}$	24.90 $^{g–l}$ (+14)	30.01 $^{b–h}$ (+37)	25.60 ^{D}
Shahrekord	0.3506 ^{ml}	0.4246 $^{h–m}$ (+20)	0.5010 $^{f–h}$ (+42)	0.425 ^{E}	17.72 ^{m}	20.73 $^{l–m}$ (+16)	24.28 $^{i–l}$ (+37)	20.91 ^{E}
Mashhad	0.4895 $^{f–i}$	0.7468 ^{ab} (+52)	0.8276 ^{a} (+67)	0.688 ^{A}	22.41 $^{j–m}$	29.50 $^{d–i}$ (+32)	31.81 $^{b–f}$ (+41)	27.91 ^{CD}
Avg.	0.3968 ^{C}	0.5302 ^{B}	0.6399 ^{A}		23.85 ^{C}	29.32 ^{B}	33.29 ^{A}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1). § Averages of rows (main effect of genotype) and columns (main effect of irrigation regime) for each trait followed by the same uppercase letter(s) are not significantly different at the 5% probability level.

The range of MDA content was from 17.72 (nmol.g) in Shahrekord to 28.86 (nmol g) in Afghanistan under I_1. Lower MDA content within genotypes suggests the greater antioxidative activities alongside resistances to arid conditions [61]. In this regard, Ghadyeh-Zarrinabadi and colleagues showed that, under drought, the MDA content in pot marigold increased in drought-sensitive genotypes [23].

Under stress conditions, unsaturated fatty acids in cell membranes are impacted by free radicals, resulting in a chain reaction of lipid peroxidation. The quantity of malondialdehyde is commonly referred to as a lipid peroxidation marker [62]. H_2O_2 is a relatively long-lived molecule, making it easier to quantify in tissue samples. H_2O_2 can oxidize thiol groups in enzymes, such as those involved in the Calvin cycle, copper/zinc superoxide dismutase, and iron superoxide dismutase [63]. Different antioxidative enzymes in plant cells may have a role in regulating the H_2O_2 levels within the cell. The MDA and H_2O_2 levels increased dramatically when there was a water constraint, but the rise varied depending on the genotype and irrigation regime. Moreover, Wang and colleagues reported an increase in the MDA content under water stress in maize [64], and Maghsoodi and collaborators in alfalfa [41].

In our study, the H_2O_2 content was observed to increase from +8% in Semirom to +54% in Syria under I_2, and +25% in Semirom to +95% in Syria under I_3 as compared with I_1. Under all the irrigation regimes, Mashhad also had the highest H_2O_2 content, whereas Semirom produced the lowest H_2O_2 content (Table 8). The results indicate that Semiron, based on the lowest H_2O_2, and Isfahan, based on the lowest MDA, were the more drought-tolerant genotypes.

Table 8. Analyses of variance at three irrigation regimes (I) in two years (Y).

Sources of Changes	DF	Plant Height
I	2	3241.38 **
Y	1	307.91 ^{ns}
I × Y	2	27.33 ^{ns}
Error	12	11.63
G	9	2296.89 **
G× Y	9	36.97 ^{ns}
G × I	18	148.08 **
I × Y × G	18	45. 36 ^{ns}
Total error	108	867.99

* and **: significant at the 5% and 1% probability levels, respectively; ^{ns}: non-significant.

3.6. Plant Height

The results show a significant interactive effect (at 1% probability levels) of genotype and irrigation regime on plant height in black cumin (Table 8). Plant height was shown to decline with an increasing moisture deficit in all the genotypes, but this decrease is dependent on the genotype and irrigation regime. Drought also causes a reduction in the leaf size, stem extension, stomatal conductance, and photosynthetic rate [3,15].

Other authors found, in marigold and fennel, that the extent of the reduction in plant height depended on the genotype and irrigation regimen, which is consistent with our findings [23,26,27]. In contrast, Baghalian and colleagues in *Plantago* [28] and Seyed and collaborators in black cumin [32] found that drought stress had no effect on plant height, which could be due to differing water regimes and experimental settings. In the current study, a decrease in the plant height was recorded ranging from −6% in the Afghanistan genotype to −42% in the Isfahan genotype under I_2, and from −15% in Afghanistan to −48% in Mashhad under I_3 (Table 9). However, the plant height ranged from 33 cm in India to 63 cm in Shahreza under I_1 (Table 9). Other workers have documented a similar decrease in the plant height of black cumin, fennel, and cumin in response to water stress [24,26,27].

Table 9. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on plant height of black cumin.

	Plant Height (cm)			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**
Afghanistan	36.69 $^{e–h}$ †	34.56 $^{g–i}$ (−6) ‡	31.47 ^{hi} (−15)	34.90 ^{G} §
Pakistan	36.35 $^{e–h}$	28.00 $^{j–m}$ (−23)	27.15 ^{l} (−26)	30.50 ^{EF}
Syria	35.38 $^{e–i}$	31.55 $^{g–i}$ (−5)	29.11 ^{hi} (−18)	32.01 ^{FG}
India	33.31 $^{e–i}$	30.25 ^{hi} (−10)	27.22 ^{i} (−19)	30.25 ^{D}
Arak	61.75 ^{a}	42.35 ^{de} (−32)	33.27 $^{f–i}$ (−46)	45.80 ^{A}
Isfahan	59.33 ^{a}	34.67 $^{e–i}$ (−42)	32.13 $^{g–k}$ (−46)	41.37 ^{E}
Semirom	57.18 ^{ab}	49.03 ^{d} (−15)	31.30 $^{d–g}$ (−45)	45.80 ^{CD}
Shahreza	62.93 ^{a}	48.74 $^{b–d}$ (−22)	41.79 $^{d–f}$ (−34)	59.90 ^{B}
Shahrekord	55.88 ^{a}	47.51 ^{cd} (−15)	40.54 $^{d–g}$ (−28)	48.00 ^{BC}
Mashhad	58.68 ^{a}	48.75 ^{d} (−17)	30.29 ^{hi} (−48)	45.90 ^{A}
Avg.	49.74 ^{A}	39.70 ^{B}	32.20 ^{C}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1). § The means indicated with the same capital letters in the rows (main effect of the genotype) and in the columns (main effect of the irrigation regime) for each trait are not significantly different at the 5% probability level.

3.7. Capsules and Seeds per Plant

In general, water stress also negatively affects the number of capsules formed and the number of seeds produced in black cumin (Table 10). The genotypes most sensitive to water stress are Pakistan, Syria, India, and Mashhad, which already, at the I_2 water regime, reduce the number of capsules by 52, 44, 51, and 44%, respectively. In these genotypes, the capsule production is reduced by approximately 60% at the I_3 level of water stress. The Semirom and Shahrekord genotypes are the most resistant to the two water stress regimes, maintaining reductions below 20%.

Table 10. Mean comparison of the effect of irrigation regime (I), genotype (G), and their interaction on capsules number per plant and seeds per capsule of black cumin.

	Seeds per Capsule				Capsules Number per Plant			
Genotypes	**I_1 ***	**I_2**	**I_3**	**Avg.**	**I_1**	**I_2**	**I_3**	**Avg.**
Afghanistan	14.08 $^{g–l}$	11.32 $^{i–n}$ (−20)	10.23 ^{c} (−28)	10.43 ^{F} §	57.33 $^{g–i}$ †	47.50 $^{k–m}$ (−18) ‡	35.00 ^{o} (−38)	46.27 ^{C}
Pakistan	19.31 $^{c–f}$	9.32 $^{n–p}$ (−52)	6.66 $^{o–p}$ (−60)	11.76 $^{E–F}$	72.16 ^{c}	49.16 $^{j–m}$ (−32)	38.0 ^{no} (−48)	53.22 ^{D}
Syria	19.25 $^{c–f}$	10.76 $^{j–o}$ (−44)	6.97 $^{o–p}$ (−63)	12.33 $^{D–F}$	61.00 $^{e–g}$	46.25 ^{lm} (−25)	35.00 ^{o} (−43)	47.50 ^{C}
India	21.14 ^{cd}	10.45 $^{n–p}$ (−51)	8.50 $^{n–p}$ (−60)	13.36 $^{C–E}$	76.33 ^{c}	46.04 ^{lm} (−40)	38.92 ^{no} (−49)	53.90 ^{B}
Arak	26.06 ^{ab}	22.06 $^{b–d}$ (−16)	10.61 $^{j–o}$ (−60)	19.58 ^{A}	95.0 ^{a}	59.00 ^{fh} (−38)	37.82 ^{no} (−60)	64.93 ^{A}
Isfahan	18.33 $^{d–g}$	14.01 $^{h–l}$ (−24)	11.61 $^{k–n}$ (−36)	14.65 ^{CD}	70.50 ^{cd}	51.16 $^{i–l}$ (−28)	35.50 ^{o} (−32)	52.55 ^{B}
Semirom	16.66 $^{e–h}$	14.40 $^{g–k}$ (−13)	13.85 $^{h–l}$ (−17)	14.97 ^{BC}	70.83 ^{cd}	60.16 $^{e–g}$ (−16)	55.20 $^{g–i}$ (−22)	62.11 ^{A}
Shahreza	23.46 ^{abc}	20.57 ^{cde} (−13)	13.54 $^{k–m}$ (−40)	19.17 ^{A}	85.33 ^{b}	65.33 ^{de} (−24)	43.83 ^{mn} (−48)	64.83 ^{A}
Shahrekord	15.17 $^{f–i}$	14.63 $^{g–j}$ (−4)	12.10 $^{k–n}$ (−20)	13.96 $^{C–E}$	71.66 $^{e–f}$	64.33 ^{ef} (−10)	52.00 $^{i–k}$ (−27)	62.94 ^{A}
Mashhad	26.76 ^{a}	14.79 $^{g–j}$ (−44)	10.07 $^{l–p}$ (−65)	17.21 ^{AB}	90.16 ^{ab}	54.83 $^{h–j–f}$ (−38)	43.40 $^{m–n}$ (−53)	62.61 ^{A}
Avg.	19.96 ^{A}	14.21 ^{B}	10.39 ^{C}		75.03 ^{A}	54.43 ^{B}	41.51 ^{C}	

* I_1, I_2, and I_3 = 40, 60, and 80% depletion of available soil water, respectively. † Means in columns and rows (interaction) for each trait followed by the same lowercase letter(s) are not significantly different at the 5% probability level. ‡ Values in the parentheses show the percentage increase (+) or decrease (−) of the irrigation regime compared with the control (I_1). § The means indicated with the same capital letters in the rows (main effect of the genotype) and in the columns (main effect of the irrigation regime) for each trait are not significantly different at the 5% probability level.

As expected, the number of seeds showed a notable reduction in the genus India (−40%), Arak (−38%), and Mashhad (−38%) already in the I_2 regime. Furthermore, Arak and Mashhad, when subjected to the I_3 regimen, confirmed their sensitivity to water stress as they lost more than 50% of the number of seeds. There is no loss of more than 27% of the number of seeds in the Semirom and Shahrekord genotypes, thus confirming their resistance to water stress. All these data confirm the close relationship between water stress and seed production. Plants that are more resistant to drought do not greatly reduce the number of capsules and seeds because they have already developed systems of tolerance to arid environments [65]. On the contrary, the less resistant genotypes have to implement survival strategies of the new individuals (seeds). Indeed, the production of capsules and seeds requires an enormous energy effort for the plant. The reduction of water triggers the responses of the mother plant with the aim of preserving the future progeny. The lesser quantity of water means less availability of resources and less chance of survival. Therefore, reducing the number of seeds (and/or capsules) will increase the availability and the chances of survival [65].

3.8. Stress Susceptibility Index and Principal Component Analysis

In the current study, our data indicate a significant relationship between the activity of antioxidant enzymes and the stress susceptibility index (SSI), which demonstrates the role of these enzymes in drought-tolerance in black cumin genotypes (Table 11). Furthermore, our findings suggest that Shahrekord under I_2 and Semirom under I_3 are the preferred genotypes for drought tolerance. The drought-tolerant genotypes were shown to exhibit a greater antioxidant enzyme activity. These data suggest that more drought-tolerant genotypes protect their cells more efficiently against damage by free radicals with a reduced yield loss, whilst drought-sensitive genotypes (i.e., Mashhad and Pakistan) are characterized by lower antioxidant enzyme activity and a higher MDA content.

Table 11. Rankings of ten black cumin genotypes based on the stress susceptibility index of seed yield under irrigation after 60% depletion of available soil water (I_2) and under irrigation after 80% depletion of available soil water (I_3).

Stress Susceptibility Index under I_3 (SSI_3)				Stress Susceptibility Index under I_2 (SSI_2)		
Genotypes	**SSI_2**	**Ranking**	**Group**	**SSI_3**	**Ranking**	**Group**
Afghanistan	0.63	2	Tolerant	0.989	4	Moderate
Pakistan	1.562	10	Susceptible	1.184	10	Susceptible
Syria	1.252	8	Susceptible	1.118	8	Susceptible
India	1.379	9	Susceptible	1.1	6	Susceptible
Arak	0.746	5	Moderate	1.118	7	Susceptible
Isfahan	0.748	6	Moderate	0.748	3	Tolerant
Semirom	0.723	4	Tolerant	0.586	1	Tolerant
Shahreza	0.713	3	Tolerant	1	5	Moderate
Shahrekord	0.572	1	Tolerant	0.621	2	Tolerant
Mashhad	1.247	7	Susceptible	1.137	9	Susceptible

A principal component analysis (PCA) was performed for the different traits studied (Figure 1). The cosine of the angles between the vectors indicates how closely the variables are related. Positive correlations are represented by acute angles (90°), while negative correlations are characterized by wide, obtuse angles (>90°). The length of the vectors linking the traits to the origin reveals the level of variability and contribution of each trait in the PCA. The intensity of the correlation increases for angles approaching 0° and 180°. The two components justified more than 87% of the variation under severe stress (I_3). Under severe stress, a significant and positive correlation between APX, CAT, Chl-a, and Chl-b with leaf proline content and soluble carbohydrates was observed. In particular, a positive and significant correlation between the Chl contents and the activity of antioxidant enzymes was observed (Figure 1). Based upon the least reduction and highest retainment of Chl content under drought, it appears that, of those tested, Semirom was the most drought-tolerant genotype, possibly due to its higher RWC under drought conditions (see Table 4). Moreover, the PCA results indicate a positive correlation between proline content and antioxidant enzyme activity, as well as a negative relationship with MDA (Figure 1). Indeed, based on the highest proline content under all the irrigation regimes, Shahrekord was shown to be the most drought-tolerant genotype. Moreover, there was a positive and significant correlation between leaf proline content and soluble carbohydrates with RWC. It seems that these osmolytes, as proline and carbohydrates, may induce a reduction in the water stress in both the Shahrekord and Semirom genotypes. All the aforementioned traits were shown to exhibit a significant negative correlation with H_2O_2, MDA, and SSI. A significant and negative correlation was shown between stress susceptibility index (SSI) and the activity of antioxidant enzymes, TSC, and proline content. These data also indicate that there is a highly significant negative relationship between H_2O_2 and MDA with the activity of antioxidant enzymes; but a significant and positive correlation was observed between H_2O_2 and MDA with SSI. Therefore, the H_2O_2 and MDA contents may be used as phenotypic markers for the future selection and breeding of drought-tolerant black cumin genotypes.

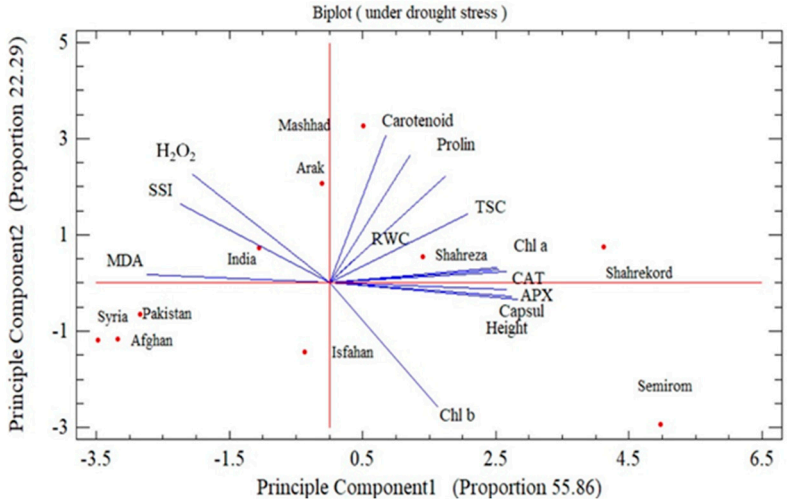

Figure 1. Projection (axis 1 and 2 of a principle component analysis) of physiological, biochemical, and morphological traits of black cumin under water stress (I_3) condition.

4. Conclusions

This study revealed that the level of irrigation markedly influenced the physiological, biochemical, and morphological features of the black cumin genotypes studied, although the impact/affect upon these parameters varied according to the genotype, irrigation regime, and the trait in question. Water stress was clearly shown to increase the activities of CAT and APX and the carotenoid content, proline, soluble sugars, MDA, and H_2O_2 but reduced the RWC and chlorophyll content. Based upon the proline, TSC, RWC, H_2O_2, MDA, and SSI, the black cumin genotypes were classified into drought-tolerant ('Shahrekord' and 'Semirom'), semi-tolerant ('Arak', 'Mashhad', and 'Shahreza'), semi-sensitive ('India' and 'Isfahan'), and drought-sensitive ('Pakistan', 'Syria', and 'Afghanistan') groups under I_3 (Figure 2). According to the data presented, Mashhad under I_1 and Arak under I_2, and Semirom under I_3 could be recommended for maximum yield production. Based on the stress susceptibility index, the genotypes Shahrekord under I_2 and Semiron under I_3 were shown to be the most drought-tolerant.

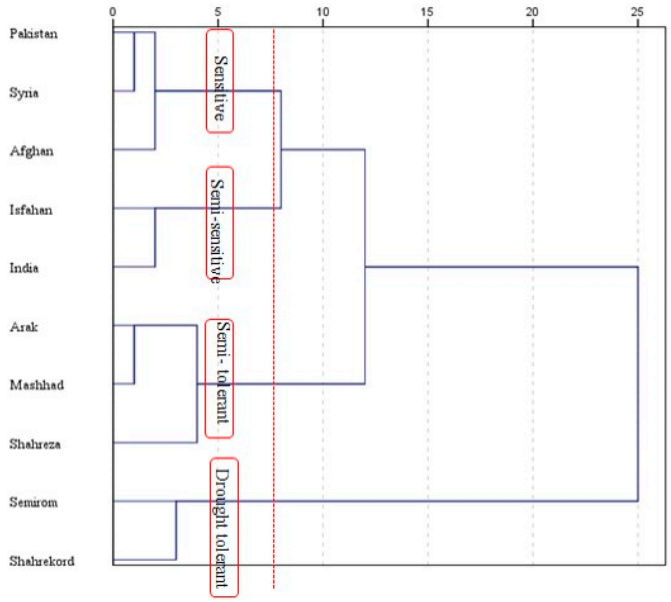

Figure 2. Dendrogram of 10 genotypes of black cumin based on the means of proline, TSC, RWC, H_2O_2, MDA, and SSI. Data collected from 2017 to 2018 and using Ward clustering.

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