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Joint Effects of Developmental Stage and Water Deficit on Essential Oil Traits (Content, Yield, Composition) and Related Gene Expression: A Case Study in Two *Thymus* Species

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Abstract: In this study, the joint effects of developmental stage and soil water availability on biomass accumulation, harvest index, as well as essential oil content, yield and composition were investigated in *Thymus armeniacus*. For comparison purposes, *Thymus kotschyanus* was also considered. Plants were irrigated to either 75 or 50% of field capacity, and were sampled at 50 or 100% blooming. In both species, water deficit exerted limited effect on the time required to initiate or complete flowering. In most critical aspects of yield (harvestable organs dry weight, essential oil yield), *T. armeniacus* was found to be superior than *T. kotschyanus*. In these traits, however, *T. armeniacus* underwent a more drastic water deficit-induced decrease. Across treatments, metabolite levels fairly correlated to transcript accumulation profiles of terpene synthases and cytochrome P450 genes. Indices affiliated with reactive oxygen species were inter-correlated with the activity of five major antioxidant enzymes, while the same was noted between leaf water status and pigment content. Taken together, these results indicate that when water availability can be achieved, higher yields will be obtained by cultivation of *T. armeniacus*. Under water deficit conditions, instead, the more drought tolerant *T. kotschyanus* stands out as the primary choice.

Keywords: blooming; harvest index; terpene; *Thymus armeniacus*; *Thymus kotschyanus*; water deprivation

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1. Introduction

In medicinal and aromatic plants, cultivated sources are increasingly gaining ground over wild populations [1]. This shift is underlain by many factors, including consistency of supply and alignment of product standards to market regulations and consumer preferences. Depending on the intended market, herbal material may be traded fresh or dried. Alternatively, the focus may be on the production of essential oils, and in this regard essential oil yield is of interest [2]. Since the composition of essential oil determines its properties, it also sets essential oil quality and market value [3]. Therefore, adjustments

in cultivation protocols may be required to improve herbal material (fresh and dry) yield, essential oil yield or essential oil composition.

The dissemination of *Thymus* spp. (Lamiaceae) is centered in the Mediterranean area, where several species of the genus are also widely cultivated [4]. An extensive interspecific variation in every aspect of production has been documented. Although an important amount of information and knowledge has been gained, it is limited to certain species. Instead, data on other taxa is not publicly available for industrial use. For instance, both *Thymus kotschyanus* Boiss. and Hohen. and *Thymus armeniacus* Klokov and Des.-Shost. are widely cultivated in Iran. However, contrary to *T. kotschyanus* [5], data on biomass accumulation, essential oil yield and composition is essentially absent in *T. armeniacus*. In this perspective, there is a great demand of developing and establishing cultivation protocols.

The developmental stage at harvest is a factor affecting essential oil production. In some *Thymus* species, for instance, essential oil production is considerably affected by flowering induction, whereas flowering exerts a less pronounced effect in other taxa [6,7]. Therefore, the importance of harvesting time associated with essential oil features varies depending on the species of interest.

Although *Thymus* species are generally well adapted to water deficits, limited soil water availability may decrease productivity and alter essential oil composition [8]. These effects are underlain by a diverse range of processes. For instance, essential oil is highly concentrated in leaf trichomes, the density of which is affected by water deficit depending on the species. Another illustration is the water deficit-induced reduction in chlorophyll content, which impedes carbon assimilation. Furthermore, the water deficit elicits osmotic stress, impairing the activity of several enzymes and damaging the structure of critical macromolecules [2]. These effects may be effectively alleviated by the accumulation of specific osmolites (e.g., proline) [2]. In addition, plants under water deficit conditions generally experience an imbalance between the generation and scavenging of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POX), polyphenol oxidase (PPO), and superoxide dismutase (SOD) are key ROS detoxification enzymes, while carotenoids and polyphenols are major non-enzymatic antioxidant metabolites [9–11]. Excessive ROS generation elicits a range of harmful effects, including lipid peroxidation [9,10,12].

The objectives of this study were to investigate for the first time the dual effects of harvesting time and soil water availability on herbal material marketable yield (leaves and flowers), as well as on essential oil yield and composition in *T. armeniacus*. For comparison purposes, *T. kotschyanus* was cultivated at the same stand and considered. To gain a more fundamental insight, several underlying processes were further investigated, including leaf trichome density, chlorophyll content, proline content, membrane lipid peroxidation and seven critical antioxidant defense elements. By studying the regulation of genes participating in the terpenoid biosynthetic pathway across developmental stages and species, the genetic-metabolic crosstalk was also elucidated.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of two *Thymus* species (*T. kotschyanus*, *T. armeniacus*) were sterilized, and then rinsed with distilled water. Seeds were then planted in a seedling tray (30 × 18 × 5 cm) filled with peat and perlite (9:1, *v*/*v*; Meegaa substrates BV, Rotterdam, The Netherlands). At the cotyledon stage, seedlings were transferred to 6 L pots (dimensions of 30 × 25 cm) containing the same mixture. Following sufficient growth (~six weeks), a single plant per species was selected as the mother plant. Experimental plants were further obtained by cutting propagation. For each species, branch cuttings were collected from the same (mother) plant, and were thus considered identical clones.

Following six weeks of cultivation, cuttings were fully rooted. Rooted cuttings with uniform height (~15 cm) and architecture were selected for potting. These were transplanted

to 6 L pots (dimensions of 30 × 25 cm) containing a mixture of soil, sand, and animal manure (4:2:1, *v*/*v*/*v*). Pots were transferred in a multi-span plastic greenhouse, which was located to Shahrekord (32°19′32″ N 50°51′51″ E). A density of four plants m^{-2} was employed. The air temperature was set to 24 °C, and relative air humidity to 70%. Supplementary light was set at 250 μmol m^{-2} s^{-1} photosynthetic photon flux density at the top of the plant canopy for 16 h d^{-1}, and was provided by red (90%) and blue LED modules (Iraneon Co., Birjand, South Khorasan, Iran). Potting-media moisture was maintained by regular watering. With the dual purpose of inducing the same initial status among individuals and of ensuring that herbal material growth would take place under experimental conditions (described below), all plants were pruned at 5 cm above root-to-shoot interface.

Immediately afterwards, three factors [2 species (*T. kotschyanus*, *T. armeniacus*) × 2 irrigation levels (full, and deficit irrigation) × 2 sampling stages (50, and 100% blooming)] were applied as a factorial experiment based on a completely randomized design. Irrigation was adjusted daily to 75 and 50% of field capacity, representing full and deficit irrigation, respectively. Under these irrigation schemes, substrate volumetric water content corresponded to circa 8–10 and 5–7%, respectively. These irrigation levels were selected based on growth reduction in a preliminary study. Irrigation to 100% of field capacity was not performed in order to avoid potential adverse effects related to waterlogging. Given daily irrigation and the large substrate volume (6 L), day-to-day variation in substrate moisture content is expected to be rather minimal. The irrigation scheme was maintained until the final harvest, which was conducted at the 100% blooming developmental stage (15 August 2019). Experimental plants were collected for evaluation at two developmental stages (50 and 100% blooming), based on the percentage of branches bearing open flowers. When half of the branches had open flowers, it was considered as 50% blooming. Accordingly, when all branches were in that state, it was considered as 100% blooming.

Plants were cultivated for three months (15 May–15 August 2019). During this period, the mean air temperature was 23.8 ± 1.6 °C, while the mean relative air humidity was 66 ± 7%. The average daily light integral was 20.6 ± 0.3 mol m^{-2} d^{-1} (LI-250A, LI-COR, Lincoln, NE, USA).

Plant and leaf level measurements were conducted. For leaf-level measurements, leaves were selected from the upper (toward the apex) one-third of the leaf-bearing nodes. Sampled leaves had grown under direct light, and were fully expanded. In all evaluations, three replicates were assessed per treatment.

2.2. Growth, Flowering and Harvest Index

The time required for initiation of flowering, as well as for reaching the two developmental stages (50 and 100% blooming) under study was recorded. The inflorescence height and the number of flowers per inflorescence were also documented.

The length of five branches (from the root-to-shoot junction to the apical meristem) per plant was recorded and averaged to compute plant height.

By considering the overhead (top-view) 2D plant silhouette, a convex hull (the minimal polygon that encloses the entire silhouette perimeter) [13] was fitted. The maximum and the minimum distance spanned by pairs of points crossing the convex hull center were considered as plant length and width, respectively. Based on these data, canopy area ($\pi \times \frac{\text{plant length}}{2} \times \frac{\text{plant width}}{2}$) was computed.

For individual leaf trait assessment, leaves were scanned (HP Scanjet G4010, Irvine, CA, USA) and then evaluated by using the Digimizer software (version 4.1.1.0, MedCalc Software, Ostend, Belgium) [14]. Leaf length (midvein length; major axis), width (widest point perpendicular to the leaf major axis) and area (one-sided surface area) were digitally assessed [13,15].

Aerial plant and shoot (fresh and dry) masses were recorded. For measuring dry weight, samples were placed in a forced-air drying oven for 72 h at 80 °C. Economic yield was considered the combined mass of leaves and flowers (thus excluding shoots). Harvest index ($\frac{\text{economic yield}}{\text{plant dry weight}} \times 100\%$) was also computed.

2.3. Essential Oil Content

The essential oil content is as important as essential oil yield, since it determines the extraction cost and, therefore, the profitability of essential oil production [2]. Shade-dried samples were subjected to hydro-distillation using a Clevenger apparatus [3]. These (20 g) were added to a 1 L flask containing 200 mL of distilled water. The flask was then heated for 3.5 h. Essential oil content was recorded. The isolated essential oils were first dried over anhydrous sodium sulfate, and then kept in glass vials at −20 °C before further analysis [3]. Three replicates were assessed per sample.

2.4. Essential Oil Composition

Gas chromatography-mass spectrometry (GC–MS) analysis was conducted using a gas chromatograph (Model 7890A, Agilent, Palo Alto, CA, USA) coupled with a mass selective detector (Model 5673, Agilent, Palo Alto, CA, USA). A fused silica capillary column (30 m length × 0.25 mm i.d.; 0.25 µm BP-5 film thickness) was used to separate the oil compounds. The oven temperature was increased from 40 to 290 °C (at a rate of 7 °C min^{-1} for 40–200 °C, and of 40 °C min^{-1} for 200–290 °C), and finally held isothermal at 290 °C for 10 min. Ion source and transfer-line temperature was 290 °C. Ultra-pure helium was used as the carrier gas. Injector and interface temperatures were 290 °C and 280 °C, respectively. The mass spectrum was acquired over the mass range of 35–450 amu in full-scan acquisition mode. The split ratio was 1:50.

The GC-FID analysis of the essential oils was conducted using a Thermoquest Finnigan apparatus equipped with a flame ionization detector (FID) and a fused silica capillary column (30 m length ×0.25 mm i.d.; 0.25 µm BP-5 film thickness). The oven temperature was programmed as stated above. Injector and detector temperatures were 250 °C and 300 °C, respectively. Ultra-pure helium was used as the carrier gas with a flow rate of 2.1 mL min^{-1}. The split ratio was 1:10.

Retention indices (RI) of each compound were calculated using a homologous series of n-alkanes (C_6–C_{24}) injected to HP-5MS column in the same condition. Identification of oil constituents was performed by comparison of (1) their retention times with those of authentic standards, (2) their spectral mass with those of the internal reference mass spectra library (NIST08 and Wiley 9.0), and (3) their RI with those reported in the literature [16]. Quantification was conducted by the external standard method through calibration curves generated by running GC analysis of representative authentic compounds [3]. The relative percentage of each essential oil constituent was obtained according to the respective area under the curve by using the area normalization method and ignoring response factors [3].

2.5. Leaf Trichome Density

Glandular trichomes secrete essential oils. In this perspective, treatment effects on glandular trichome density of either leaf side were studied. The sampling area (1 × 1 cm) was located midway between the leaf base and tip, and between the midrib and lateral margin. Images were acquired using an optical microscope (Leitz Aristoplan; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) connected to a digital camera (Nikon DXM-1200; Nikon Corp., Tokyo, Japan). Glandular trichome density was counted on five non-overlapping fields of view per leaf (magnification × 100). Image processing was performed with ImageJ software (https://imagej.nih.gov/).

2.6. Leaf Water Status

Leaf water status was in situ assessed by measuring relative water content (RWC). Samples were collected 3 h following the onset of the photoperiod [17]. Following excision, fresh weight was gravimetrically obtained (±0.0001 g; Mettler AE 200, Giessen, Germany). Immediately after, samples were floated on distilled water inside a Petri dish covered with a lid. Following 24 h of incubation, the recorded weight was regarded as turgid (saturated). Then, dry weight (48 h at 80 °C) was determined. RWC was calculated according to Taheri-Garavand et al. [18].

2.7. Leaf Chlorophyll and Carotenoid Content

Leaf chlorophyll content is critical for photosynthesis, while carotenoids are important non-enzymatic antioxidants [1,11]. Samples were processed immediately after collection. Following fine chopping, portions weighing 0.1 g were homogenized with the addition of 10 mL of 100% acetone. The extract was then centrifuged (14,000× *g* for 20 min), and the supernatant was collected. Since chlorophyll is light sensitive, the extraction took place in a dark room [9,10]. The obtained extract was subjected to reading on a spectrophotometer (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China). Total chlorophyll and carotenoid contents were calculated according to Lichtenthaler and Wellburn [19].

2.8. Leaf Proline Content

Proline is actively involved in cell osmotic regulation via decreasing cell water potential, and in this way enzyme activity and the macromolecules' structure are protected [2]. In this perspective, the treatment effect on leaf proline content was assessed. Freshly cut leaf discs (0.5 g) were homogenized and then added in 10 mL of 3% (*w*/*v*) aqueous sulphosalycylic acid. The extract was filtered through Whatmann No. 2 filter paper, and 2 mL of the filtrate were mixed with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid. The obtained solution was heated (100 °C for 1 h). The reaction mixture was extracted with 4 mL toluene, and the chromophore containing toluene was aspirated from liquid phase. After equilibration at 25 °C, the absorbance was measured at 520 nm with a spectrometer (Mapada UV-1800, Shanghai. Mapada Instruments Co., Ltd., Shanghai, China). Proline concentration was determined using a calibration curve [20].

2.9. Leaf Hydrogen Peroxide Content

H_2O_2 is a critical ROS which accumulates under adverse conditions [10]. Leaf H_2O_2 content was, therefore, assessed. The reaction mixture consisted of tissue extract supernatant, 0.5 mL 0.1% trichloroacetic acid, 0.5 mL of 0.1 M potassium-phosphate buffer (pH 7.0), and 1 mL of 1 M KI (*w*/*v*). Color developed for 45 min in darkness, and absorbance was then spectrophotometrically assessed at 390 nm (UV-1800, Shimadzu, Kyoto, Japan). H_2O_2 content was calculated by using a calibration curve prepared with eight known H_2O_2 concentrations.

2.10. Leaf Lipid Peroxidation

The treatment effects on the malondialdehyde (MDA) content, taken as an indication of lipid peroxidation level, were evaluated by employing the thiobarbituric acid reactive substance assay [2]. Freshly-cut leaf discs (0.1 g) were homogenized, and then added in 5 mL of 20% (*w*/*v*) trichloroacetic acid and 0.5% (*w*/*v*) thiobarbituric acid. The suspension was subsequently centrifuged (6000× *g* for 15 min). The obtained solution was heated (100 °C for 25 min). After equilibration at 25 °C, the precipitate was removed by centrifugation (6000× *g* for 5 min). The amount of MDA was calculated from the absorbance at 535 nm after subtracting the non-specific absorption at 450 and 600 nm (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China). The extinction coefficient 156 mmol MDA L^{-1} cm^{-1} was used. Four discs were assessed per replicate sample.

2.11. Antioxidant Defense Elements

2.11.1. Total Phenolic Content

As phenolics exhibit strong antioxidant properties, they can be beneficial for plant antioxidant defense [12]. Leaf total phenolic content was therefore assessed. Leaf samples (0.1 g) were ground with a mortar and pestle with liquid nitrogen, extracted with 1 mL of 80% aqueous methanol in an ultrasonic bath for 10 min, and were then centrifuged (15,000× *g* for 10 min). The content of total phenolics was determined by using the Folin-Ciocalteu assay [12]. The absorbance against prepared re-agent blank was determined using a microplate reader (Infinite 200 PRO, TECAN, Männedorf, Switzerland). For total

phenolic content, gallic acid was used as the standard reference and gallic acid equivalent (GAE) was expressed as mg per g dry mass [12].

2.11.2. Activity of Five Antioxidant Enzymes

The treatment effects on the activity of five critical antioxidant enzymes (APX, CAT, POX, PPO, SOD) was assessed.

APX activity was assessed using the method described by Ahmadi-Majd et al. [9,10]. Fresh frozen leaf segments (0.1 g) were ground in liquid nitrogen, homogenized with 1 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 1% polyvinylpyrrolidone (PVP), and centrifuged (14,000× *g* for 20 min) at 4 °C. APX activity in the supernatant was assessed by following the decrease in absorbance at 290 nm for 2 min (10 s intervals) in a reaction mixture containing sodium phosphate buffer, ascorbic acid, and H_2O_2. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used. APX activity was expressed as µmol of ascorbate oxidized min^{-1} g^{-1} tissue.

CAT activity was measured as described by Ahmadi-Majd et al. [9,10]. Fresh frozen leaf segments (0.3 g) were ground in liquid nitrogen, homogenized with 1.5 mL of K phosphate buffer (containing 1 mM EDTA and 2% PVP), and centrifuged (14,000× *g* for 20 min) at 4 °C. CAT activity in the supernatant was assessed by following the decrease in absorbance at 240 nm for 2 min (10 s intervals) in a reaction mixture containing potassium phosphate buffer and H_2O_2. The extinction coefficient of 39.4 M^{-1} cm^{-1} was used. CAT activity was expressed as µmol of H_2O_2 reduced min^{-1} g^{-1} tissue.

SOD activity was determined by the method of Ahmadi-Majd et al. [9,10] and was assayed by monitoring the inhibition of the photochemical reduction of nitro-blue tetrazolium chloride (NBT). Fresh frozen leaf segments (0.5 g) were ground in liquid nitrogen, homogenized with 1 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 1% PVP, and centrifuged (14,000× *g* for 20 min) at 4 °C. A reaction mixture of sodium phosphate buffer, methionine, NBT, EDTA, and riboflavin was used. The mixture was placed for 20 min at 25 °C under a fluorescent light (30 Watt). Absorbance at 560 nm was monitored using a spectrophotometer (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China). A SOD enzyme activity unit was considered as 50% of the NBT photoreduction and expressed as unit min^{-1} g^{-1} tissue.

PPO activity was determined according to Chen et al. [11], using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 0.15 mL supernatant was mixed with 0.6 mL phosphate buffer (25 mM) and 0.15 mL catechol (20 mM), and incubated for 10 min at 25 °C. It was then was immediately placed into boiling water for 10 min and cooled to room temperature (25 °C). Next it was centrifuged (10,000× *g* for 10 min) at 25 °C. Thereafter, using distilled water as a blank, the absorbance was measured at 410 nm with a microplate reader (Infinite 200 PRO, TECAN, Switzerland). One unit of PPO activity was defined as the amount of enzyme required to increase the absorbance by 0.01 min^{-1} under the assay conditions.

POX activity was performed according to the method of Narwal et al. [21]. The reaction mixture contained 1350 µL of 0.1 M potassium phosphate buffer (pH 6), 500 µL of 44 mM H_2O_2 solution, 100 µL of 45 mM guaiacol and 200 µL of extracted enzyme extract. Absorbance at 470 nm was monitored using a spectrophotometer (Mapada UV-1800; Shanghai Mapada Instruments Co., Ltd., Shanghai, China).

2.12. QPCR Expression Analysis

Total RNA from each sample was extracted using the total RNA isolation kit (S-1010-1, Dena Zist Asia, Mashhad, Iran). The genomic DNA was eliminated by DNAse I treatment according to the manufacturer's instructions (ThermoFisher, Waltham, MA, USA). Subsequently, first strand cDNA was amplified using M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase and oligo (dT)18 primer, from 2 µg total RNA to 20 µL final volume (Cat. No. 18080-044; Invitrogen, Carlsbad, CA, USA).

All the RT-qPCR reactions were performed using RT-Master Mix (Cat. No. RR820L, Takara Bio, Shiga, Japan) containing SYBR Green on a Rotor Gene Q (Qiagen, Hilden, Germany). The following PCR profile was employed: (1) 95° C for 30 s (pre-denaturation step), (2) 95 °C for 15 s (denaturation step), (3) 56–57 °C for 20 s (annealing), and eventually (4) 72 °C for 25 s (extension). This sequence was repeated for 35 cycles. Then a melting curve (55–95 °C) was performed at the default ramp rate. The primers efficiency was acquired from calibration curves with 1:5 dilution series and at least three points fitted in a linear regression with R-square over 0.98.

The primer used in this study was designed based on gene sequences in the NCBI database and primers designed in previous reports (Crocell et al., 2010). The accuracy of sequences and size of primers were checked using the Primer-BLAST software of NCBI (Supplementary Table S1). The qRT-PCR analysis was carried out with elongation factor-1α (EF-1α) gene as the internal standard and was analyzed using the $2-\Delta\Delta CT$ method [22].

2.13. Statistical Analysis

Data analysis was performed using the SPSS software (version 23; SPSS Inc., Chicago, IL, USA). A three-way ANOVA was employed (species × irrigation level × sampling stage). The data were first tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). The means were compared using Fisher's least significant difference test to be significant at $p \leq 0.05$.

For the four experimental units, eigenvalues were extracted and the most contributing variables for each dimension were computed and identified. The first two eigenvalues cumulated more than 77% of the total variance, and were retained to produce the principal components. A biplot principal component analysis (PCA) was produced to depict correlations across the traits, species and contributions to principal components. Individuals were grouped (by discrete color) and variables by their contribution to the principal components (gradient colors). The "FactoMineR", "factoextra" and "readxl" libraries were used under the R-studio integrated development environment (RStudio suite V 1.2.5033).

3. Results

3.1. Growth, Flowering and Harvest Index

The onset of flowering occurred much earlier in *T. armeniacus* as compared to *T. kotschyanus* (49.3 versus 79.3 d), though time to 100% blooming was rather similar among them (115–120 d; Table 1). *Thymus armeniacus* had shorter (47.6%) inflorescence length, and a lower (34.6%) number of flowers per inflorescence. Water deficit (50 as compared to 75% field capacity) reduced (~5%) time to 100% blooming similarly among the two species. In both species, the water deficit also decreased inflorescence length, and the number of flowers per inflorescence. The latter was more decreased in *T. armeniacus*, while the former in *T. kotschyanus*.

In both species, water deficit decreased plant height, length, and individual leaf dimensions (length, width, area) (Table 2). For plant height and individual leaf dimensions, this effect was less prominent in *T. armeniacus*.

Table 1. Effect of irrigation regime on time to flowering (initiation, 50 and 100%) and two key inflorescence characteristics of two *Thymus* species. The latter was evaluated at 100% flowering. Within each column, different letters indicate significant differences (n = 3).

Species	Irrigation (% of Field Capacity)	Time (d)			Inflorescence Length (cm)	Number of Flowers per Inflorescence
		Onset of Flowering	50% Flowering	100% Flowering		
T. armeniacus	75	49.3 c	63.0 c	120.3 a	2.31 b	50.22 c
	50	47.7 c	66.3 c	113.0 b	1.64 c	33.84 d
T. kotschyanus	75	79.3 b	88.0 b	114.7 ab	4.41 a	76.78 a
	50	88.0 a	96.0 a	108.7 b	2.34 b	63.07 b

Table 2. Effect of irrigation regime on plant and individual leaf dimensions of two *Thymus* species. Assessments were conducted at 100% flowering. Within each column, different letters indicate significant differences (n = 3).

Species	Irrigation (% of Field Capacity)	Plant			Canopy Area (cm^2)	Individual Leaf		
		Height (cm)	Length (cm)	Width (cm)		Length (mm)	Width (mm)	Area (mm^2)
T. armeniacus	75	14.6 bc	35.3 a	31.0 a	858 a	12.9 b	5.64 a	47.4 b
	50	13.1 c	28.8 bc	28.3 ab	640 b	11.2 c	4.89 b	41.9 c
T. kotschyanus	75	20.3 a	31.3 b	27.3 b	670 c	18. 5 a	4.71 b	57.1 a
	50	15.9 b	28.02 c	29.0 ab	637 d	14.0 b	3.74 c	38.1 c

Thymus armeniacus had higher plant fresh weight, dry weight, economic yield (dry weight of leaves and flowers), and harvest index (dry weight of leaves and flowers relative to plant dry weight) as compared to *T. kotschyanus* (111.6, 95.0, 122.7, and 14.3%, respectively; Table 3). In both species, water deficit decreased plant fresh weight, dry weight, economic yield, and harvest index. For plant fresh weight and economic yield, this effect was more pronounced in *T. armeniacus*.

Table 3. Effect of irrigation regime on plant (fresh and dry) weight, economic yield (dry mass of leaves and flowers) and harvest index ($\frac{\text{economic yield}}{\text{plant dry weight}} \times 100\%$) of two *Thymus* species. Assessments were conducted at 100% flowering. Within each column, different letters indicate significant differences (n = 3).

Species	Irrigation (% of Field Capacity)	Plant			Harvest Index (%)
		Fresh Weight (g)	Dry Weight (g)	Economic Yield (g)	
T. armeniacus	75	116.93 a	42.37 a	34.1 a	81.97 a
	50	83.78 b	32.2 b	23.71 b	76.63 b
T. kotschyanus	75	55.26 c	21.73 c	15.313 c	71.71 c
	50	42.81 d	16.413 d	12.167 c	63.99 d

3.2. Essential Oil Content, Yield and Composition

Thymus armeniacus had lower (49.9%) essential oil content, and higher (13.2%) essential oil yield as compared to *T. kotschyanus* (Table 4). The water deficit decreased (49.9%) the essential oil content in *T. kotschyanus*, whereas it exerted a minor effect (1.4%) in *T. armeniacus*. The water deficit decreased essential oil yield in both taxa, an effect that was more prominent in *T. armeniacus* (30.5 versus 24.6%).

Table 4. Effect of irrigation regime on essential oil content and yield, as well as on leaf trichome density of two *Thymus* species. Assessments were conducted at 100% flowering. Within each column, different letters indicate significant differences (n = 3).

Species	Irrigation (% of Field Capacity)	Essential Oil Content (%)	Essential Oil Yield (g)	Adaxial	Abaxial	Leaf (Adaxial + Abaxial)
				Trichome Density (mm^{-2})		
T. armeniacus	75	1.180 c	0.3870 a	4.75 c	5.640 c	10.38 c
	50	1.164 c	0.2689 b	18.1 a	12.74 a	30.84 a
T. kotschyanus	75	2.353 a	0.3420 a	8.88 b	10.32 b	19.2 b
	50	1.988 b	0.2578 b	10.0 b	11.65 ab	21.67 b

By using gas chromatography–mass spectrometry, essential oil composition was further analyzed (Table 5). For *T. armeniacus*, the most abundant metabolites were γ-Terpinene (16.65–23.58%), p-Cymene (6.70–16.56%) and α-Pinene (12.16–15.78%). The former two (i.e., γ-Terpinene, p-Cymene) were also the most abundant metabolites in *T. kotschyanus*

(17.69–28.11 and 4.19–17.78%, respectively), whereas α-Pinene was not detected in this species. In *T. kotschyanus*, thymol was also a very abundant metabolite (11.41–15.73%).

The water deficit affected substance content depending on the metabolite and the species (Table 5; full range of documented compounds in Supplementary Table S2).

Hierarchical clustering of essential oil components revealed that these could be organized primarily based on species and secondarily on irrigation treatments (Figure 1). However, neither growth stage nor irrigation regime severely distorted essential oil fractions.

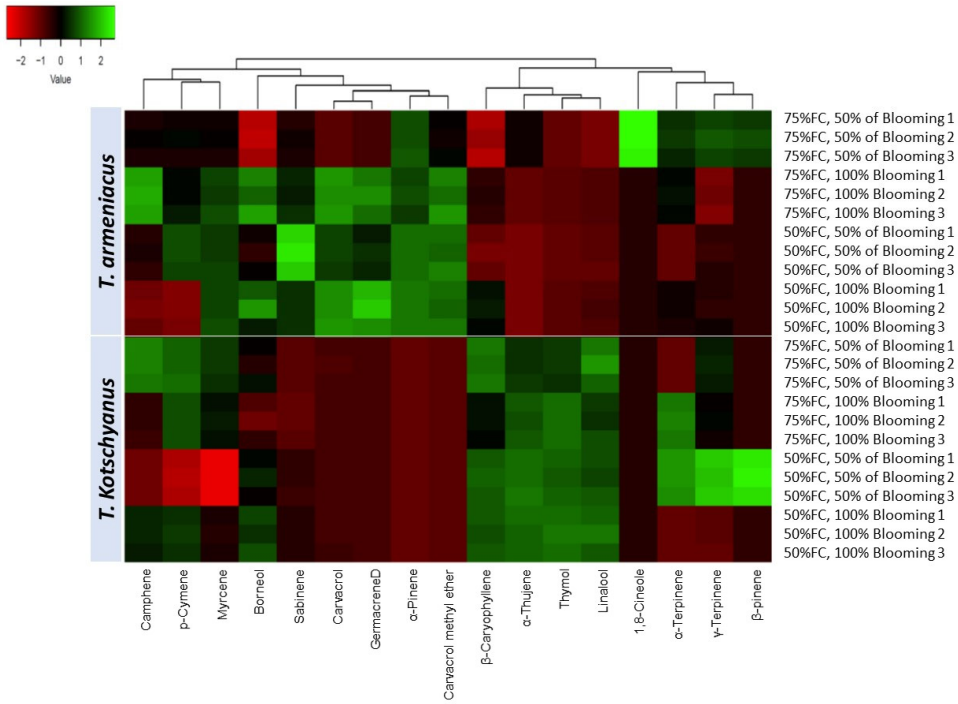

Figure 1. Heatmap of standardized values indicating percentages of essential oil components across treatments. The effect of irrigation regime (75 or 50% of field capacity) on essential oil composition of two *Thymus* species (*T. armeniacus*, *T. kotschyanus*) was investigated at two developmental stages (50 and 100% blooming). Essential oil composition is presented in Table 5. Color may indicate up- or downregulation (green and red, respectively). A scale of intensity is provided.

3.3. Principal Component Analysis in Plant Growth and Yield Traits

In order to identify and quantify the components that regulate the connections among the two *Thymus* species, as well as the effect of irrigation regime to the delineation of differential responses, a biplot PCA was conducted (Figure 2). In this analysis, all the growth traits were included (data in Tables 1–4 excluding trichome density). Eigenvalues were examined to determine the number of considered principal components (PC). The first two dimensions explained more than 77% of the total variance percentage (Supplementary Figure S1). The level of significant contribution of morphological traits to the PCA was estimated by using the cos2 index (Supplementary Figure S2). Among these descriptors, the plant fresh and dry weights as well as the economic yield and flowering/inflorescence indices had a profound impact for the categorization of species and treatments. The biplot PCA based on the first two components revealed the complex relationships among genotypes and treatments (Figure 2). The first axis revealed that *T. armeniacus* and *T. kotschyanus* can be clearly demarcated based on genotypic discrepancies under control conditions (75% of field capacity), while the second axis displays the discriminating effects imposed by water deficit within the same species. Furthermore, positive and negative correlations across morphological components were evident. Flowering and inflorescence indices were highly homogenous (indicating co-regulation) as were the plant fresh weight, dry weight and economic yield. Interestingly, the above-mentioned clusters had an inter-negative association showing that the earliness in blooming is an index of high biomass yield.

Table 5. Effect of irrigation regime on essential oil composition of two *Thymus* species at two developmental stages (50 and 100% blooming). Within each column, different letters indicate significant differences (n = 3). Retention indices (RI) were generated with a standard solution of n-alkanes (C_6–C_{24}) on the HP-5MS column. nd—no difference. The full range of documented compounds is provided in Supplementary Table S2.

Compound	RI	*T. armeniacus*				*T. kotschyanus*			
		75% Field Capacity		50% Field Capacity		75% Field Capacity		50% Field Capacity	
		50% Flowering	100% Flowering	50% Flowering	100% Flowering	50% Flowering	100% Flowering	50% Flowering	100% Flowering
α-Thujene	928	5.76 e	0.903 f	0 g	0 g	9.35 d	11.337 c	12.893 a	12.34 b
Camphene	934	3.62 d	8.12 a	3.13 e	1.34 g	7.19 b	2.82 f	1.307 g	4.883 c
α-Pinene	934	12.987 b	12.157 c	15.42 a	15.78 a	0 d	0 d	0 d	0 d
β-pinene	967	2.26 b	0 c	0 c	0 c	0 c	0 c	5.623 a	0 c
Sabinene	971	4.4067 d	6.42 c	12.43 a	6.873 b	2.347 f	2.137 f	3.71 e	4.06 de
Myrcene	988	6.1 d	8.483 ab	8.34 ab	8.623 a	8.13 b	7.22 c	0 e	5.73 d
α-Terpinene	1018	4.63667 c	3.53 d	0 f	2.623 e	0 f	7.1 b	7.797 a	0 f
p-Cymene	1034	12.067 e	13.507 d	16.56 b	6.703 f	17.783 a	16.63 b	4.187 g	15.267 c
1,8-Cineole	1037	12.53 a	0 b	0 b	0 b	0 b	0 b	0 b	0 b
γ-Terpinene	1073	23.583 b	16.65 h	19.21 f	19.84 e	21.93 c	20.74 d	28.11 a	17.69 g
linalool	1105	0 d	0.28 c	0.187 c	0.29 c	1.463 a	1.1 b	1.213 b	1.347 a
Borneol	1168	0.87 f	2.78 a	1.847 d	2.54 ab	1.927 d	1.48 e	2.067 cd	2.37 bc
Carvacrol methyl ether	1248	0.88 c	2.417 a	2.187 b	2.153 b	0	0	0	0
Thymol	1298	0.11 f	0.577 de	0.32 ef	0.66 d	11.41 c	15.66 a	14.337 b	15.73 a
Carvacrol	1313	0.827 d	11.483 a	7.657 b	11.34 a	1.45 c	1.653 c	1.52 c	1.78 c
β-Caryophyllene	1429	2.31333 f	5.22 d	3.803 e	6.693 c	8.917 a	6.52 c	8.3 b	8.193 b
Germacrene D	1484	nd	0.923 b	0.48 c	1.197 a	0 d	0 d	0 d	0 d

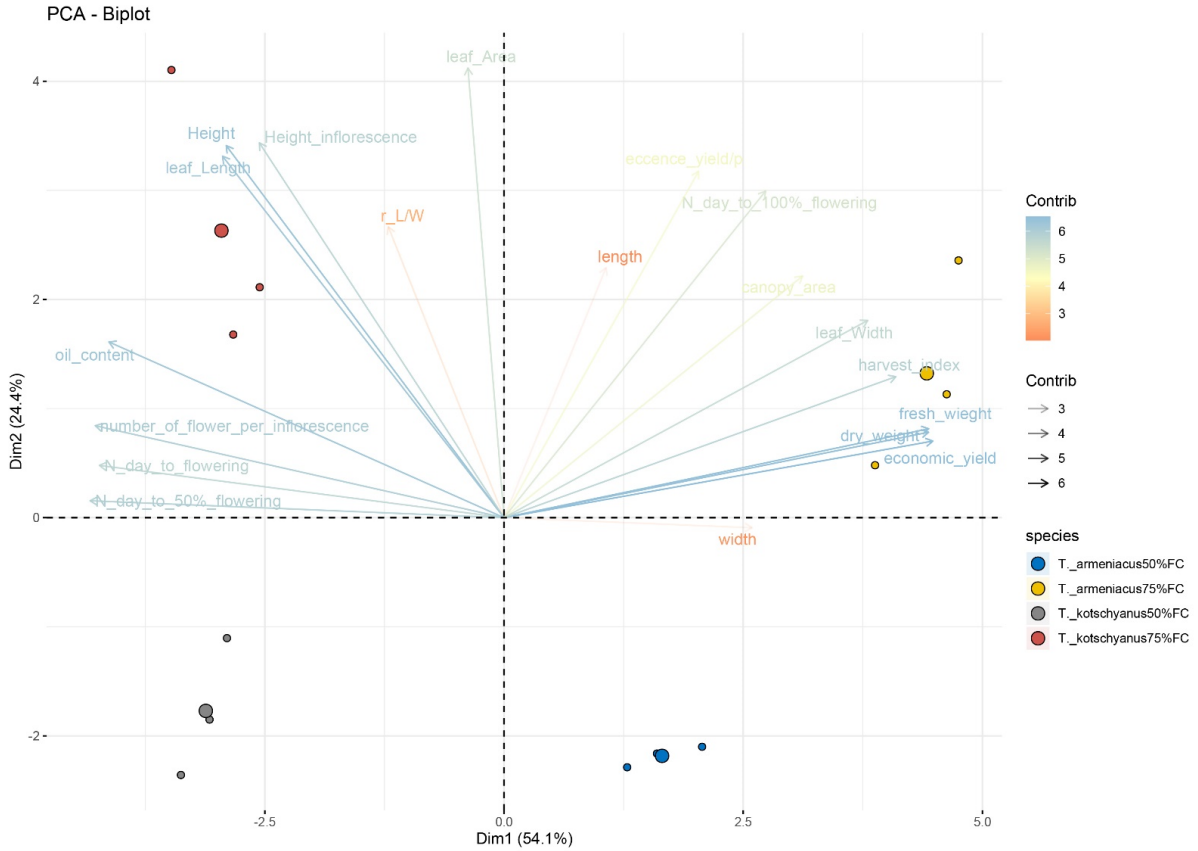

Figure 2. Principal Component Analysis (PCA) biplot depicting Principal Components (PC) scores of *Thymus* species and treatments (dots) and loadings of vector variables. The contribution of each morphological trait (data in Tables 1–4) in the two dimensions is indicated by a gradient scale and colour intensity (legend). Vectors near the plot center have lower cos2 values. Larger dots indicate mean values calculated from three discrete biological replications.

3.4. Leaf Trichome Density

Thymus armeniacus had lower (~45%) trichome density in either leaf side as compared to *T. kotschyanus* (Table 4). Most trichomes were generally situated on the abaxial leaf side as compared to the adaxial one, besides *T. armeniacus* cultivated under water deficit.

The water deficit increased trichome density in either leaf side of both species (Table 4). This increase was considerably more prominent in *T. armeniacus* as compared to *T. kotschyanus* (126–281% versus 13%).

3.5. Leaf Water Status

Leaf RWC was determined as an indication of hydration status. In both species, water deficit impaired leaf hydration status at 100% blooming (Table 6). This effect was more prominent in *T. kotschyanus*, as compared to *T. armeniacus*.

3.6. Leaf Chlorophyll, and Proline Contents

In *T. armeniacus*, the water deficit improved leaf chlorophyll content at either developmental stage (Table 6). In *T. kotschyanus*, the water deficit decreased (21.7%) leaf chlorophyll content at 50% blooming, but not at 100%.

At 100% blooming, water deficit induced a sizeable increase (~1500%) in leaf proline content (Table 6). This effect was comparable among the two species.

3.7. Leaf Hydrogen Peroxide Content and Lipid Peroxidation Level

H_2O_2 is a critical ROS. At either developmental stage, water deficit increased (~95%) leaf H_2O_2 content in both species (Table 6). This effect was comparable among the two species.

Table 6. The effect of irrigation regime on the content of chlorophyll, carotenoids, total phenolics, proline, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2), as well as on relative water content and five antioxidant enzymes activity of two *Thymus* species at two developmental stages (50 and 100% blooming). Within each column, different letters indicate significant differences (n = 3). APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; dw, dry weight; fw, fresh weight; GAE, gallic acid equivalent; POX, guaiacol peroxidase; PPO, polyphenol oxidase; SOD, superoxide dismutase; U, unit.

Trait		*T. armeniacus*				*T. kotschyanus*			
		75% Field Capacity		50% Field Capacity		75% Field Capacity		50% Field Capacity	
		50% Flowering	100% Flowering	50% Flowering	100% Flowering	50% Flowering	100% Flowering	50% Flowering	100% Flowering
Chlorophyll content (mg g^{-1} FW)	a	0.261 b	0.31 a	0.22 c	0.23 c	0.278 b	0.28 b	0.226 c	0.269 b
	b	0.09 abc	0.099 a	0.082 bc	0.086 abc	0.086 bc	0.094 ab	0.058 d	0.078 c
	total	0.351 c	0.409 a	0.302 de	0.316 d	0.364 bc	0.374 b	0.285 e	0.348 c
Non-enzymatic	Carotenoid content (mg g^{-1} FW)	2.611 c	3.444 a	1.596 e	2.611 c	2.55 c	3.085 ab	2.171 d	2.769 bc
	Total phenol content (mg GAE g^{-1} DW)	-	31.422 b	-	26.513 c	-	34.718 a	-	31.108 b
	Relative water content (%)	74.97 b	68.794 c	66.394 c	55.621 d	76.681 b	83.144 a	74.467 b	57.048 d
Osmolite	Proline content (µmol g^{-1} FW)	0.515 cd	0.378 d	0.839 bcd	6.9 a	0.921 bc	0.491 cd	1.156 b	7.088 a
	MDA content (nmol g^{-1} FW)	10.938 f	12.874 e	12.018 ef	18.707 b	12.565 e	16.355 c	14.598 d	22.323 a
	H_2O_2 content (nmol g^{-1} FW)	478.57 d	587.52 c	663.09 b	1154.65 a	422.8 e	350.25 f	702.41 b	677.79 b
Enzymatic	APX activity (µmol AsA min^{-1} mg^{-1} protein)	0.4641 de	0.3968 e	0.5707 cd	0.6533 bc	0.7366 b	0.4886 de	0.9265 a	0.6066 c
	CAT activity (µmol H_2O_2 min^{-1} mg^{-1} protein)	5.446 f	6.576 de	9.805 a	8.43 b	7.105 cd	6.23 e	7.901 b	7.735 bc
	POX activity (U mg^{-1} protein min^{-1})	34.561 c	35.915 c	86.375 b	163.423 a	35.928 c	33.459 c	94.706 b	92.627 b
	PPO activity (U mg^{-1} protein min^{-1})	0.556 e	0.666 d	0.882 b	0.974 ab	0.771 c	0.678 cd	0.962 ab	0.988 a
	SOD activity (U mg^{-1} protein min^{-1})	11.9416 b	6.553 d	13.987 a	9.941 c	7.797 d	7.663 d	9.975 c	10.350 c

MDA content was assessed as an indication of lipid peroxidation level [2]. At 100% blooming, the water deficit induced an increase in leaf MDA content (Table 6). This increase was more prominent in *T. armeniacus* as compared to *T. kotschyanus* (45.3 versus 36.4%).

3.8. Enzymatic and Non-Enzymatic Antioxidant Defense Elements

Carotenoids and polyphenols are critical non-enzymatic antioxidants, while APX, CAT, POX, PPO, SOD are important antioxidant enzymes.

In *T. armeniacus*, water deficit improved leaf carotenoid content at either developmental stage (Table 6). In *T. kotschyanus*, instead, no effect on carotenoid content was noted.

Water deficit decreased leaf total phenolic content in both species (Table 6). This effect was more prominent in *T. armeniacus* as compared to *T. kotschyanus* (15.6 versus 10.4%).

Different effects of the water deficit were noted among enzymes, species and developmental stages (Table 6).

3.9. Interplay of Physiological Traits

In order to delineate the interaction responses of each species to the watering regime across the two developmental stages, a hierarchical clustering was performed (Figure 3). In this analysis, all the physiological traits were included (data in Table 6). Two major clusters were acknowledged. The first contained oxidative indices (H_2O_2, proline and MDA levels) affiliated with ROS induction, as well as antioxidant enzymes (APX, CAT, POX, PPO, SOD). Oxidative indices were inter-correlated with antioxidant enzymes, suggesting a joint regulatory biosynthetic pathway. Further correlations among MDA and proline contents, as well as among H_2O_2 content and POX enzyme activity, were determined, suggesting a related biochemical connection. The second cluster contained the RWC index as well as chlorophyll and carotenoid contents.

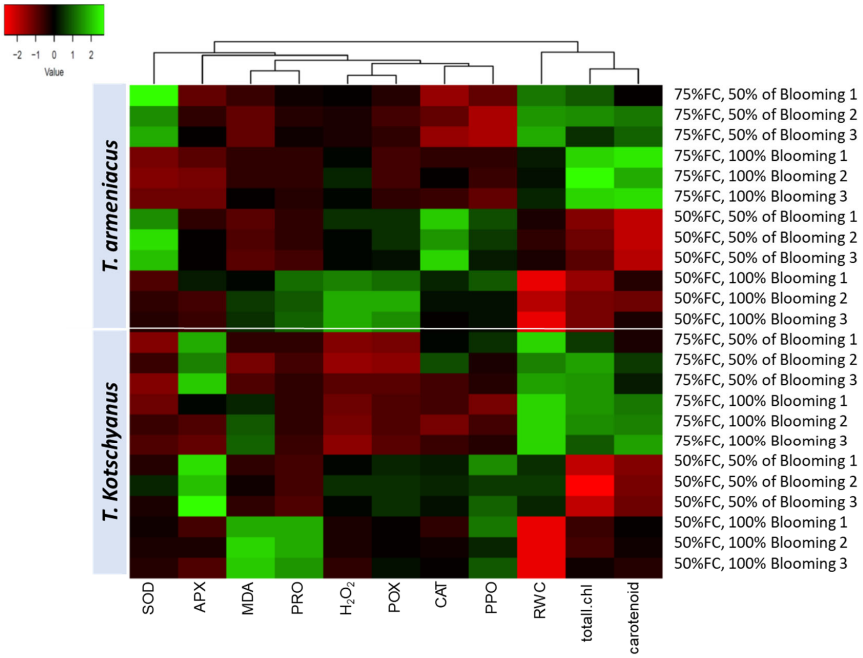

Figure 3. Heatmap of standardized values indicating physiological trait regulation across treatments. The effect of irrigation regime (75 or 50% of field capacity) on several physiological traits (data in Table 6) of two *Thymus* species (*T. armeniacus*, *T. kotschyanus*) was investigated at two developmental stages (50 and 100% blooming). Color may indicate up- or downregulation (green and red, respectively). A scale of intensity is provided.

Similar patterns across physiological traits were recorded among the two species under study (Figure 3). Specifically, a tight upregulation under water deficit that was mostly unaffected by the developmental stage, was demonstrated. Still, there were some

instances where the species effect was more prominent and several discrepancies among the two species were recorded.

3.10. Relative Expression of Terpene Synthesis

The simultaneous analysis of transcript accumulation indicates that there are distinct developmentally regulated pathways as well as stress-induced responses (Figure 4). Moreover, gene regulation in the terpene pathway seems to follow different routes based primarily on the species. Hence, major discrepancies were detected among the two species under study. In *T. armeniacus*, a significant downregulation was evident for *TERPENE SYNTHASE 6* (*TPS6*) at the 100% blooming stage, which was not specifically correlated to the water deficit. In contrast, the same isoform seems unaffected in *T. kotschyanus*, having a rather conserved expression pattern. Genes encoding CYTOCHROME P450 (CYP) monooxygenases enzymes were also differentially regulated among the two species. *CYP71D179 182* seems to be linked to water availability, since in *T. kotschyanus* a significant upregulation was detected under water deficit regardless of the developmental stage. In *T. armeniacus*, transcription patterns fluctuated at a much lesser pace. By contrast, *TERPENE SYNTHASE 2* (*TPS2*) seems to correlate to water deficit only in *T. armeniacus*, since there was a significant upregulation under the water deficit. In *T. kotschyanus*, instead, a peak was established under adequate water supply (75% of field capacity) at the 100% blooming stage. *TERPENE SYNTHASE 3* (*TPS3*) had a complete opposite transcript accumulation among the two species. A clear developmental upregulation was established in *T. armeniacus*, while blooming progression was accompanied by a significant reduction in transcription of *T. kotschyanus*. Similar patterns were recorded during the water deficit, where in the former a notable increase of *TPS3* transcripts was established, as opposed to the latter. Nonetheless, *TERPENE SYNTHASE 4* (*TPS4*) was found to be equally responsive for both species and a general downregulation pattern was observed when compared to the adequate water supply and 50% blooming stage.

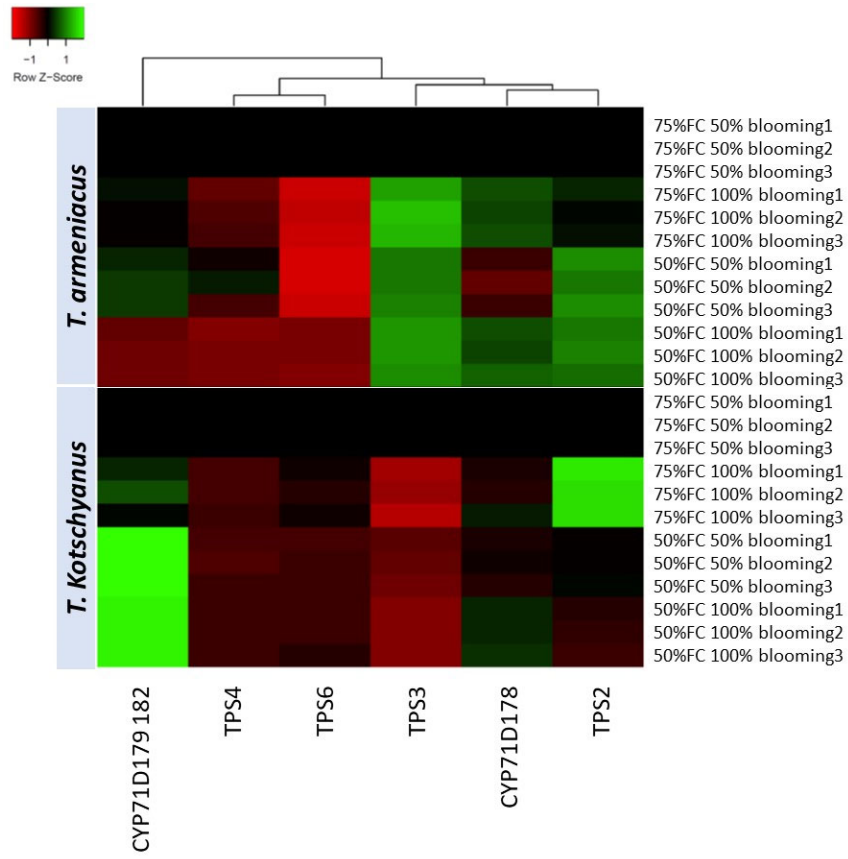

Figure 4. Heat map of relative transcript accumulation (log2) of the terpene biosynthetic pathway

across treatments. The effect of irrigation regime (75 or 50% of field capacity) on relative mRNA abundance of two *Thymus* species (*T. armeniacus*, *T. kotschyanus*) was investigated at two developmental stages (50 and 100% blooming). This was assessed using real-time RT-qPCR and three distinct biological repeats. Color may indicate up- or down regulation (green and red, respectively). A scale of intensity is provided.

4. Discussion

In Iran, *Thymus* spp. hold a wide distribution and long history of cultivation [4,23]. *Thymus kotschyanus* and *T. armeniacus* are not only highly popular amongst farmers, but they are also considered as greatly adapted for cultivation in Iran. Regardless, research has principally focused on the former [5], while information and knowledge on the yield traits of the latter is scarce. In this study and for the first time, the combined effects of developmental stage at harvest and soil water availability on several yield features were assessed in *T. armeniacus*. Evaluations included *T. kotschyanus* for comparison purposes.

Despite the little interspecies difference in the time required for full (100%) blooming, *T. armeniacus* underwent the beginning of flowering much sooner than *T. kotschyanus* (Table 1). Therefore, the overall period of blooming is considerably more extended in *T. armeniacus* as compared to *T. kotschyanus* (66 versus 41 d). Minor effects of soil water availability on flowering time were generally noted in both species under study (Table 1). Therefore, water deficit in the extent applied in the current study did not substantially affect the transition from the vegetative to the generative phase.

Thymus armeniacus developed shorter inflorescences with less flowers as compared to *T. kotschyanus* (Table 1). Both inflorescence traits were adversely affected by water deficit. Length was more decreased in *T. kotschyanus*, whereas the number of flowers per inflorescence was more impaired in *T. armeniacus* (Table 1). These results indicate that water deficit downgraded inflorescence features, though species differences were inconsistent among traits.

As discussed, yield may encompass fresh or dry herbal material, which in the case of *Thymus* spp. principally includes leaves and flowers. In all relevant aspects of yield, *T. armeniacus* was found to be superior than *T. kotschyanus* (Table 3). Nevertheless, the water deficit decreased plant fresh weight and economic yield more in *T. armeniacus* than *T. kotschyanus* (Table 3). In *T. kotschyanus*, a negative effect of drought on agronomic traits has also been earlier documented [24–26]. Complementarily or alternatively, the yield may be comprised of essential oil features [2,3]. Despite lower essential oil content, essential oil yield was higher in *T. armeniacus* than *T. kotschyanus* (Table 4). Similarly to plant fresh weight and economic yield, the water deficit decreased essential oil yield more in *T. armeniacus* than *T. kotschyanus* (Table 4). Taken together, these results indicate that when water availability can be secured, the cultivation of *T. armeniacus* will be associated with higher yield (thus returns), whereas under water deficit conditions the choice may be shifted to *T. kotschyanus*, which is more drought tolerant. In this perspective, *T. kotschyanus* bares a better potential for water limiting environments. Importantly, this potential is expected to increase in the near future, provided that global climate models project declining rainfall patterns allied with elevated temperatures [27].

Trichomes comprise highly dedicated secretory cells where most essential oils are anabolized and subsequently amassed in subcuticular cavities [28,29]. Although *T. armeniacus* had lower (46%) leaf trichome density as compared to *T. kotschyanus*, it underwent a much more drastic water stress-induced increase (197 vs. 13%; Table 4). Nonetheless, essential oil content was practically unaffected (1.33% reduction) in *T. armeniacus*, whereas a 15.48% reduction was noted in *T. kotschyanus* (Table 4). Still, under the molecular prism, a significant downregulation of terpene synthases and cytochromes was noted in *T. armeniacus* (Figure 4), which potentially reduces terpene precursors and thus leads to a decreased concentration of moieties in leaf trichomes.

Across *Thymus* taxa, over 360 discrete essential oil components have been characterized. An excessive part (>90%) of these moieties are attributable to the nature of monoterpenes. Among these derivatives, carvacrol and thymol are considered the most common [30]. In the current study, significant differences in terms of qualitative essential oil profiles across developmental stages at harvest and irrigation regimes were noted among the two species. *Thymus armeniacus'* terpene palette was mostly composed of terpinenes, although monoterpenes were positively regulated by the developmental stage, as well as the water deficit (Figure 1). In contrast, several pinene class terpenes were not detected in *T. kotschyanus,* revealing an inferior complexity of essential oil chemical markup. Nonetheless, several monoterpenes and monoterpene alcohols (thymol, linalool, a-Thujene) were found to be largely unaffected by the developmental stage at harvest or by soil water availability. Hence, at least in the case of *T. armeniacus* and *T. kotschyanus,* it seems that the main factor of chemical markup differences is predominantly species dependent.

In terms of physiological traits and ROS detoxifying responses, both species under study were affected by the water deficit (Table 6). Comparable patterns have been observed recently [31]. In some traits (MDA content, total phenolic content), *T. armeniacus* was more adversely affected than *T. kotschyanus*, while in others (chlorophyll content) the opposite was apparent. A comparable effect of water deficit among the two species was also documented on some traits (proline content, H_2O_2 content). Across treatments, metabolites affiliated with oxidative stress (H_2O_2, proline and MDA levels) were inter-correlated with antioxidant enzymes (APX, CAT, POX, PPO, SOD) (Figure 3). This correlation might be taken to indicate a combined regulatory biosynthetic pathway, which has also been earlier suggested [9–11]. Leaf hydration status (RWC) was also inter-correlated with leaf pigment (chlorophyll, carotenoid) content (Figure 3). Similar patterns have also previously recorded [8,31–33].

Since the present field study included a single growing season, additional research is evidently essential to reach solid results and conclusions. Nevertheless, the obtained findings serve both as a first step and a reference of how the cultivation protocol shapes marketable yield and the essential oil quality (composition) of a previously non-studied *Thymus* species.

5. Conclusions

For the first time, the dual effects of developmental stage at harvest and soil water availability on plant growth, marketable herbal material yield, as well as essential oil content, yield and composition were investigated in *Thymus armeniacus*. *Thymus kotschyanus* was also included for comparison purposes. Irrigation was adjusted to either 75 or 50% of field capacity, and plants were collected at 50 or 100% blooming. Water deficit did not substantially affect the time required for the transition from the vegetative to generative phase in either species. However, the water deficit decreased inflorescence length and the number of flowers per inflorescence. *Thymus armeniacus* was associated with enhanced yield (e.g., dry weight of harvestable organs, essential oil yield) as compared to *T. kotschyanus*, but was more affected by water deficit. Water deficit triggered an increase in leaf trichome density, an effect that was sizeable in *T. armeniacus*. Essential oil composition was largely unaffected by developmental stage at harvest or soil water availability. Oxidative stress indicators (proline, H_2O_2 and malondialdehyde levels) were inter-correlated with the activity of five key antioxidant enzymes, while a respective correlation was found for leaf water status and pigment (chlorophyll, carotenoid) accumulation. Collectively, these results denote that higher yields will be obtained by cultivating *T. armeniacus* when soil water is readily available. In contrast, under conditions of limited soil water availability, the choice shifts to the more drought tolerant *T. kotschyanus*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12051008/s1, Table S1. Primers used for the qPCR experiments. Table S2. Effect of irrigation regime on essential oil composition of two Thymus species at two developmental stages (50 and 100% blooming). Within each column, different letters indicate significant differences (n = 3). Retention indices (RI) were generated with a standard solution of n-alkanes (C_6–C_{24}) on the HP-5MS column. Figure S1. The first ten principal components and percentages of attributed variation. The first two eigenvalues were used to construct the principal component analysis biplot (accounting for the 77.4% of the cumulative percentage explained). Figure S2. Quality of representation (cos2) of the variables on factor map. variables on the first five dimensions are displayed. Size and colour intensity correlate to a better representation of specific morphological traits.

Author Contributions: Conceptualisation, S.H., B.S., S.M.-F. and D.F.; methodology, K.Y., S.M.-F., N.N. and D.F.; software, S.M.-F., N.N. and D.F.; validation, D.F.; formal analysis, N.N. and D.F.; resources, K.Y. and H.Z.; data curation, K.Y., S.M.-F., N.N. and D.F.; writing-original draft preparation, S.M.-F., K.Y. and D.F.; writing-review and editing, N.N. and D.F.; supervision, S.H. and B.S.; project administration, and funding acquisition, S.H., B.S. and M.M.G. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase enzyme; CYP, CYTOCHROME P450; dw, dry weight; fw, fresh weight; GAE, gallic acid equivalent; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro-blue tetrazolium chloride; PC, principal components; PCA, principal component analysis; POX, guaiacol peroxidase; PPO, polyphenol oxidase; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; RWC, relative water content; SOD, superoxide dismutase; *TPS2*, *TERPENE SYNTHASE 2*; *TPS3*, *TERPENE SYNTHASE 3*; *TPS4*, *TERPENE SYNTHASE 4*; *TPS6*, *TERPENE SYNTHASE 6*; u, unit.

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