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Research Article

Morphological and Physiological Plant Responses to Drought Stress in *Thymus citriodorus*

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Water availability is considered as a determinant factor that affects plant growth. The commercial medicinal values of an aromatic plant rely on the presence of secondary metabolites that are affected under water shortage. Two-year-old *Thymus citriodorus* plants were subjected to different polyethylene glycol (PEG-6000) levels (0, 2%, and 4%) under greenhouse condition. PEG treatment lasted for 15 days. Thyme plant showed a morphological drought avoidance mechanism by maintaining the root system development through shoot fresh weight reduction resulting in promoted root absorption capacity and sustained plant growth. Moreover, stressed plants were able to maintain water use efficiency and root : shoot ratio suggesting a strong relation between root water uptake and water use saving strategies. Furthermore, thyme plants reduced tissue dehydration through stomatal closure and improved root water uptake. Content of volatile oil constituents of geraniol and diisobutyl phthalate increased upon drought stress while pseudophytol was reduced. Unexpectedly, thymol was not reported as a main oil element under either control or mild stress condition, while it was increased upon high drought stress in measure of 4.4%. Finally, carvacrol significantly accumulated under high drought stress (+31.7%) as compared to control plants.

1. Introduction

The Mediterranean flora is well known for abundance in aromatic plants that estimated 49% of genera including aromatic species [1]. The genus *Thymus* is considered as a polymorphic one and belongs to the family Lamiaceae. It is one of the largest genera and comprises around 350 species of perennial, aromatic herbs, and subshrubs; it was found predominantly in Mediterranean region, Asia, Southern Europe, and North Africa [2]. Thyme plant is considered as one of the traditional medicinal plants largely used in folk medicine in Mediterranean area [3]. Products include oil, oleoresins, and fresh and dried herbs that can be used as antiseptic, antibacterial, and spasmolytic agents. As thyme demand increases, improving its cultivation under unfavorable environmental conditions such as drought stress will enhance its natural conservation and will sustain the pharmaceutical and beauty industries. The commercial medicinal values of aromatic plants rely on the presence of different phytochemical secondary metabolites components such as tannins, alkaloids, terpenoids, and phenolic compounds that cause particular physiological effects on the human body. Indeed, water supply is one of the most determinative cultivation conditions, which essentially affect the yield and oil content of herb crops [4]. It was reported that drought stress can be a major factor in increasing concentration of secondary metabolic products in some medicinal plants including *Hypericum brasiliense* L. [5] and *Calendula officinalis* L. [6]. However, different irrigation strategies have been reported to potentially improve aromatic plants yield. Jordán et al. [7] stated that there was no significant change in oil yield of *Thymus hyemalis* under four different watering levels, while Sotomayor et al. [8] reported that the maximum plant dry matter production and oil yield in *Thymus zygis* were achieved with a moderate watering level. Babaee et al. [9] studied the effects of four different levels of irrigation and mentioned a general reduction in all vegetative plant growth parameters such as plant height, dry and fresh weight of root and shoot, and length of roots under severe stress while thymol percentage was increased. *Thymus citriodorus* (lemon thyme or citrus thyme) is a lemon-scented variegated perennial shrub herb plant with approximate height of 50 cm and 30 cm diameter. The behavior of *Thymus citriodorus* under limited moisture availability in terms of growth and aromatic oil content has not been investigated yet. The plant is considered, as any aromatic species that are slow growing and slow reproducing, under the risk of local extinction [10]. Consistently, this research addresses the definition of a strategy for improving its medicinal and aromatic yield under drought conditions.

Drought stress causes plant dehydration, stomatal closure, and limited gas exchanges, followed by inhibition of metabolism and photosynthetic rate and finally plant death [11]. However, ability of plants to survive under stressed condition depends on plant species, growth stage, duration, and intensity of water deficit [11]. Moisture deficiency induces different plant structural changes which are critical to respond to drought stress, ranging from morphological adaptations (decline in growth rate, deep rooting system, and modification of root to shoot ratio for desiccation avoidance [12]) as well as physiological and metabolic responses (stomatal closure, antioxidant accumulation, and expression of stress specific genes). Polyethylene glycol (PEG) is a reliable marker under laboratory conditions for inducing drought stress because of its reputation as an osmotic agent resulting in increasing solute potential and blockage of absorption of water by the root system [13]. Zhang and Kirkham [14] stated that osmotic stress generated by PEG (−0.57 MPa) reduced photosynthetic rate, leaf water potential, and transpiration rate in sunflower plant. This study aims at understanding the *Thymus citriodorus* response to drought stress by examining the morphological and physiological changes on plants after PEG application and estimating the influence of water scarcity on oil biosynthesis.

2. Materials and Methods

2.1. Plant Material and Growth Conditions. Ontogenetic homogeneous two-year-old *Thymus citriodorus* plants were used to assess the physiological and morphological plant responses and evaluate the essential oil yield toward drought stress. The experiment was conducted under controlled environmental conditions (day T° 25°C; night T° 20°C; RH: 70%) in the experimental greenhouse at the University of Bologna, Italy. Every day, soil moisture content was restored by weighing all plant pots on an electronic balance (Mettler PM4600, Columbus, OH, USA). Polyethylene glycol (PEG-6000) was used as a drought agent and was added to the irrigation water in three different concentrations: 0 (control), 2% (moderated drought), and 4% (high drought stress). The experimental design used randomized blocks and each block had three replications and three plants per replicate. Plants were placed on a bench with a distance between pots of 0.2 m. Drought stress was applied on mature plants and harvesting was conducted fifteen days after drought stress (DAD).

2.2. Plant Growth Measurement. At the harvest time, morphological measurements were conducted, including shoot and root fresh (FW) and dry (DW) weights (after drying at 60°C) as well as root : shoot ratio based on dry weight. Total root area was measured by placing newly harvested roots in a rectangular polycarbonate tray (200 mm × 150 mm) with a thin layer (4 mm depth) of water to allow all roots to spread appropriately. Later, digital scanned images (EPSON V300, Suwa, Japan) were analyzed by *ImageJ* processing software [15]. The mean value of nine replications per treatment was calculated.

2.3. Leaf Gas Exchanges. Leaf transpiration (E), stomatal conductance (g_s), and net photosynthesis (A) were continuously measured at 1-day interval for 11 days starting 24 h from drought stress application. All gas exchange parameters were measured using a CIRAS-2 (PPSystem, Hitchin, UK) infrared gas analyzer (closed system) with a Parkinson's Automatic Universal Leaf Cuvette (PAR 1000 $\mathrm{mmol\,m^{-2}\,s^{-1}}$, 26°C, $\mathrm{CO_2}$ 13.63 $\mathrm{mmol\,l^{-1}}$, and 300 $\mathrm{cm^3\,min^{-1}}$ flow rate) equipped with 18 mm diameter and 1.75 $\mathrm{cm^2}$ area cuvette inserts. However, as thyme leaves are so small (4–20 mm long) and for avoiding any misleading measurements, readings were conducted by putting always 4 leaves simultaneously into the cuvette and measurements were normalized on the actual leaf area obtained by *ImageJ* processing software. Mean values were obtained from nine replications per treatment. Water use efficiency (WUE) was determined as the ratio between A and E.

2.4. Plant Nitrogen Status. A leaf chlorophyll meter N-tester (Hydro, Minolta, Osaka, Japan) was used at 15 DAD to indicate the nitrogen status of the plant. N-tester measurement was conducted on nine replications per treatment and each value represented the mean of thirty independent readings.

2.5. Relative Water Content. Relative water content (RWC) was determined on 9 individual leaves of three plant pots for each treatment at 15 DAD. Leaves were harvested and weighed to obtain the fresh weight (FW) and then placed in distilled water (4°C) and reweighed again after 24 h to obtain saturated weight (SW) [15]. Finally, leaf dry weight (DW) was determined after drying at 65°C for 48 hours and RWC was calculated as (FW – DW)/(SW – DW).

2.6. Oil Composition Compounds. At 15 DAD, dried plant material was hydrodistilled using a Clevenger type apparatus for 2 h and the average oil content was calculated on the dry weight basis (mL/100 g DW) in three replicates according to Pluhár et al. [16]. The volatile oil samples were stored in sealed vials under refrigeration prior to analysis.

2.6.1. Gas Chromatography. Samples were analyzed by gas chromatography using an Agilent Technologies 6890 N instrument (Santa Clara, CA, USA) coupled with a HP mass spectrometer. The gas chromatograph is equipped with a split-splitless injector and a Factor Four™ VF-35 ms 5% phenyl-methylpolysiloxane, 30 m, 0.25 mm, and 0.25 μm film thickness capillary column. The programmed temperature of the capillary columns includes a range of 60°C to 240°C at 3°C min^{-1}. The injector was maintained at a temperature of 250°C. The inert gas was helium at a flow of 1.0 mL/min and the injected volume in the splitless mode was 1 μL.

2.6.2. Mass Spectrometric Analysis. GC-MS analyses were carried out using an Agilent Technologies 6890 N GC equipped with an Agilent Technologies MS 5975 detector and an ionization energy of 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms. The linear retention indices were calculated using the generalized equation of Van den Dool and Kratz [17]. The MS and linear retention indices (LRI) were compared with data of NIST and home-made libraries to the identification of the essential oil compounds.

2.7. Data Analysis. Data was subjected by one-way ANOVA analysis and the means were compared using LSD at $p \leq 0.05$. All data are presented as means ± SD of nine replications.

3. Results

3.1. Plant Vegetative Growth Responses. PEG application resulted in significant differences between treatments ($p = 0.0026$) on shoot fresh weight, where the treated plants showed a reduction by 20% and 29%, respectively, at moderate and high PEG levels as compared to control plants (Figure 1). However, there were no effects of PEG on either root fresh weight ($p = 0.4999$) or root area developments ($p = 0.2515$) (Figure 1) and root dry weight ($p = 0.3543$) (data not shown). Likewise, root : shoot ratio showed no significant effect of PEG between different treatments ($p = 0.3029$) (data not shown).

3.2. Plant Physiological Responses

3.2.1. Relative Water Content (RWC). The extent of physiological stress caused by inclusion of PEG into the growth media was assessed by measuring the relative water content (RWC). Application of 2% and 4% of PEG altered significantly the plant water status ($p = 0.0000$) and showed RWC reduction by −24% and −65%, respectively, as compared to control plants (Figure 2).

3.2.2. Nitrogen Plant Status. The imposition of PEG caused a significant reduction in total chlorophyll content by −29% and −81%, respectively, under mild and high drought stress (Figure 2).

3.2.3. Gas Exchange Parameters. Gas exchange parameters were analyzed at 24 hours after stress initiation and were measured continuously for 11 days at 1-day interval of PEG application. Generally, drought stress induced by PEG-6000 promoted significant differences between treated and non-treated plants in their water relation and stomatal behaviors. The stomatal conductance (g_s) showed fast declines within the initial 48 h of water stress (data not shown); however, 4% and 2% of PEG application on the day 11th of drought showed g_s significant reduction by −62% and −35% compared to the control plants (Figure 3). Indeed, the g_s decline was also associated with an essential reduction in net assimilation rate (A) in measure of −40% and −50% under moderate and high drought stress conditions (Figure 3). Furthermore, the measurement of water loss, in terms of transpiration rate (E), revealed a significant reduction in E values by −53% and −27% compared to control plants (Figure 3). Nevertheless, PEG exposure did not affect the water use efficiency (WUE) between different treatments ($p = 0.2772$) (Figure 3).

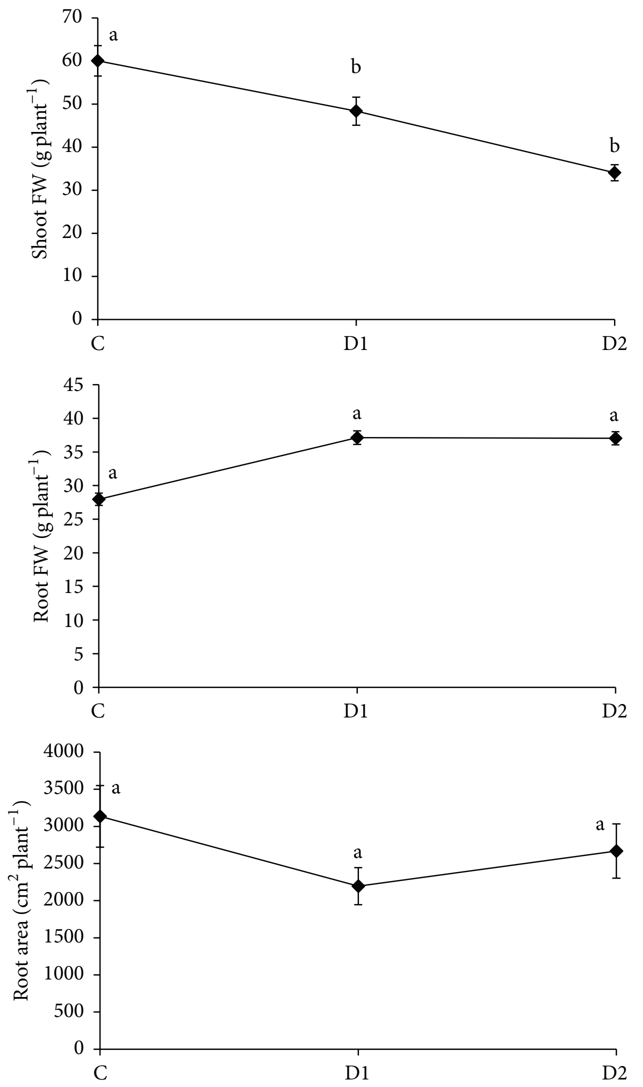

Figure 1: Effects of PEG levels (0, C; 2%, D1; and 4%, D2) on shoot and root fresh weight (FW) and root area of *Thymus citriodorus*. Values are mean ± SD of nine replicates.

3.2.4. Yield of Oil Components. The oil profile differed widely in the nature of volatile presented in the oil leaf extract

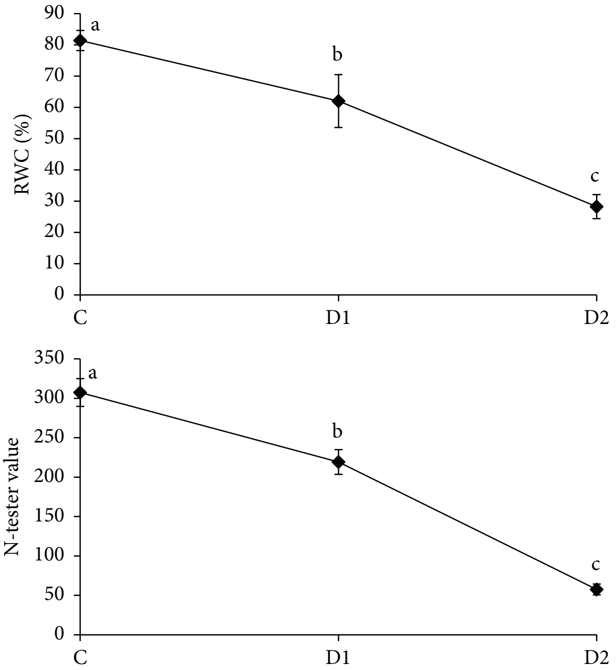

Figure 2: Effects of PEG levels (0, C; 2%, D1; and 4%, D2) on relative water content (RWC) and leaf chlorophyll content expressed as N-tester values of *Thymus citriodorus*. Values are mean ± SD of nine replicates.

in response to drought (Figure 4 and Table 1). In general, the oil yield was limited and independent from the stress (10 μL g^{-1} DW) (Figure 4). However, a significant reduction in oil content was recorded with higher level of PEG-treated plants (6 μL g^{-1} DW) (Figure 4).

Seven major oil compounds have been identified in this chemotype of thyme as follows: geraniol, geranyl butyrate, thymol, carvacrol, diisobutyl phthalate, β-caryophyllene, and pseudophytol (Table 1). One of the most abundant volatile organic compounds that has been detected in plant was diisobutyl phthalate that constitutively showed 26.3% of the main oil components, while its percentage increased to 55.1% under mild drought stress. Among the oxygenated monoterpenes, geraniol was the only oil component that increased significantly upon drought stresses (28.4% and 21.9%, resp., versus 4.2% in the control plants). Variation in the monoterpenoid phenolic components such as carvacrol and thymol was influenced by drought where carvacrol content was significantly increased in 4% PEG-stressed plants by 31.7% compared to its negligible amount in the control and mildly stressed plants. However, thymol has not been quoted in this work as a main oil element and it was presented in a trace amount under control or mild stress conditions, while it showed a pronounced increasing upon high drought stress (+4.4%). Pseudophytol accounted for 12.1% of oil components in nontreated plants and accumulated to less extent under mild and high drought stress (8.5% and 5.0%, resp.). β-caryophyllene as a sesquiterpene hydrocarbon was undetected under control condition while it showed a significant increasing (+2.1%) at high drought stress. Finally, geranyl butyrate was in a trace amount in all treatments.

Table 1: Percentage of selected oil components of *Thymus citriodorus* plant undergoing different PEG levels (0, C; 2%, D1; and 4%, D2). RT = retention time of compound; LRI = linear retention index.

Oil components	RT	LRI	C	D1	D2
Geraniol	17.20	1252	4.2	28.4	21.9
Thymol	18.81	1290	0.0	0.0	4.4
Carvacrol	19.20	1300	0.0	0.5	31.7
β-caryophyllene	23.68	1420	0.0	1.4	2.1
Geranyl butyrate	29.33	1566	0.0	1.1	1.0
Diisobutyl phthalate	40.31	1878	26.3	55.1	30.6
Pseudophytol	47.67	2064	12.1	8.5	5.0

4. Discussion

4.1. Plant Vegetative Growth Response. Drought stress is considered as one of the most important growth-limiting factors that decrease plant growth. Injury of plants takes place under water shortage at any vegetative growth stage [18]. Simulation of drought stress by polyethylene glycol (PEG) induces drought stress on the plants and significant deviation from control continues to increase with the increasing solute potential [14]. In the presented study, shoot FW of PEG-treated plants showed an averaged significant reduction (−25%) compared to the control ones (Figure 1). One explanation of this reduction could be that plants grown in nutrient culture containing PEG have a large viscosity, and so there is a possibility that a boundary layer of oxygen-depleted solution may form around the root and accordingly the plant may have suffered from hypoxia [19]. A previous study of Said-Al Ahl et al. [20] attributed the reduction in herbage yield in oregano and chamomile upon water stress to the diminishing in photosynthesis and plants canopy structure. Singh et al. [21] reported that some plants show a water stress adaptive mechanism by restricting their growth while water shortage in the rhizosphere persists. Accordingly, we might suggest that thyme plants conserve their growth and sacrifice the vegetative development for gaining a longer term survival under drought stress condition.

Well-developed root system is considered as a strategy for desiccation avoidance in natural vegetation. Morphological root components play a major role in root hydraulics that can reflect different responses to drought stress [22]. In the recent work, there was no effect of PEG implementation on the root area and its fresh (Figure 1) and dry weights (data not shown). However, our results were consistent with Bramley et al. [22] who mentioned that a large root system is not always beneficial to shoot water use, since greater root system would consume more photosynthetic end products for their growth and affect shoot growth during water shortage. This reduction might indicate that more competitive carbohydrate was allocated to roots resulting in greater decline in shoot yield than roots in order to maintain root function and promoting the root absorption capacity to enhance the plant

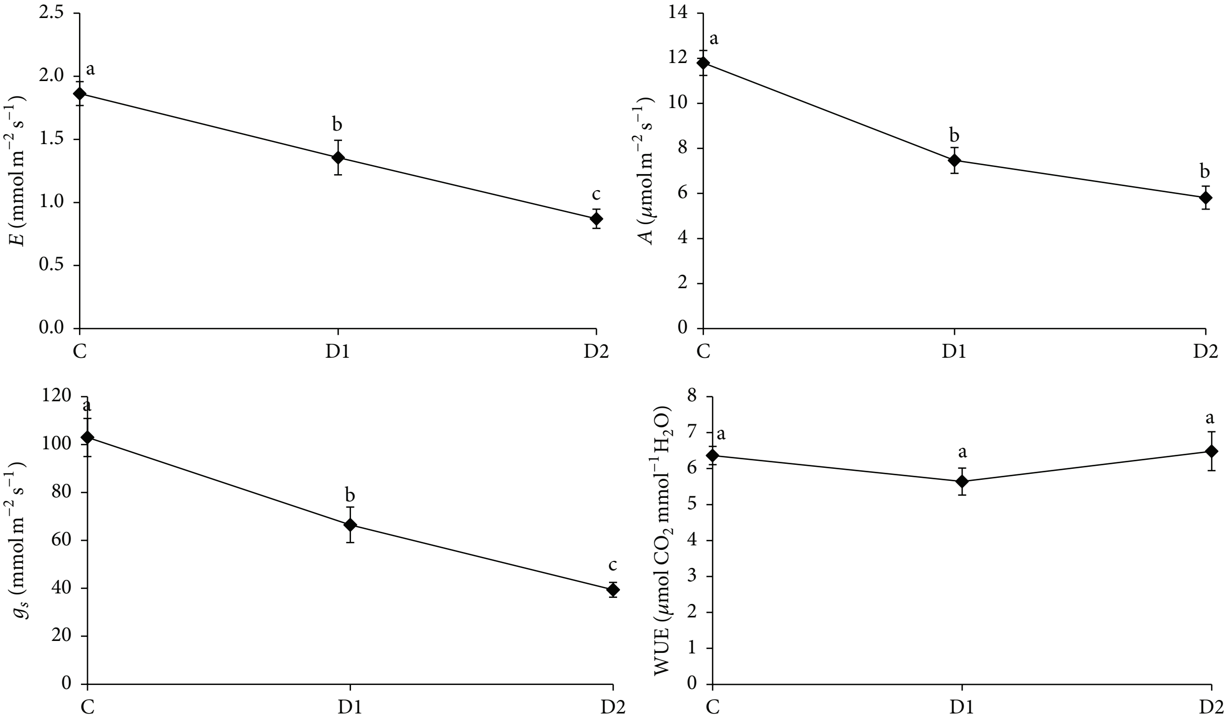

Figure 3: Effects of PEG levels (0, C; 2%, D1; and 4%, D2) on transpiration rate (E), stomatal conductance (g_s), net photosynthesis (A), and water use efficiency (WUE) of *Thymus citriodorus*. Values are mean ± SD of nine replicates.

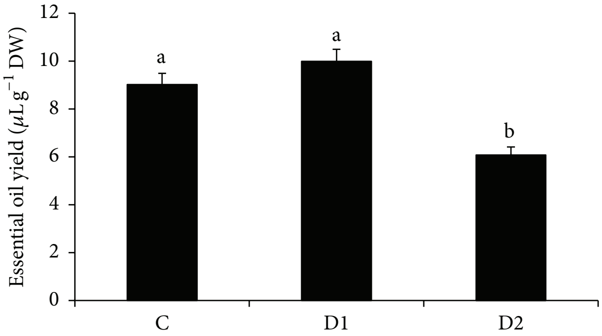

Figure 4: Effects of PEG levels (0, C; 2%, D1; and 4%, D2) on the oil yield of *Thymus citriodorus*.

growth [23]. Thus, we might suggest that thyme plant showed a drought avoidance mechanism by maintaining the root system development through shoot fresh weight reduction which has also enhanced plant water uptake.

The most challenged condition of water stressed plants is how to share the carbon assimilation for both root formation and shoot vegetative growth. It is definitely proved that the increasing root : shoot ratio is considered one of the avoidance mechanisms enabling plants to maximize the water uptake under drought stress condition [24]. In our investigation, PEG application did not result in any significant change on root : shoot ratio (data not shown). Previous report from Dodd et al. [25] mentioned that this ratio was indirectly affected by ABA concentration. Under water shortage, ABA biosynthesis will increase in the root and, successively, the synthesized ABA will be transported to the shoot through the xylem; however, the higher concentration of ABA in root may also limit the function of ethylene which accordingly inhibits the plant growth (Figure 1). Subsequently, the reduction in canopy size in thyme plant might be considered as a determinant factor in increasing the root : shoot ratio.

4.2. Plant Physiological Responses

4.2.1. Plant Water Status, Chlorophyll Content, and Gas Exchange Parameters. RWC is probably the most appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. It expresses the relative amount of water present in the plant tissues. In this research, drought stress significantly reduced RWC from 80% under control condition to 62% and 28%, respectively, under both stress levels (Figure 2). The reduction in RWC indicated a loss of turgor that resulted in limited water availability for cell expansion process and subsequently suppressed plant growth development (Figure 1). A previous study from Blokhina et al. [26] by microscopic investigations of dehydrated plant cells revealed the presence of cell membrane cleavage and its increasing permeability under drought stress condition. They mentioned that the concentrations of appropriate solutes that could preserve membrane were not sufficient and the plant was not able to adjust osmotically.

Investigation on the nitrogen plant status using N-tester showed a significant reduction in leaf chlorophyll content at PEG application in range of 29% and 81%, respectively, at mild and high drought stress levels compared to the control plants (Figure 2). Drought stress has been reported to reduce both chlorophyll content and biosynthesis [27]. A reason for chlorophyll content reduction is that drought stress enhances the production of reactive oxygen species (ROS) such as O_2^- and H_2O_2 that can lead to lipid peroxidation and, consequently, chlorophyll degradation [28].

Plants grown in water stress conditions showed alteration in cell carbon metabolism, which is possibly mediated by low CO_2 availability [29]. It is known that the presence of PEG-6000 causes primarily reduction in root water uptake and then rapid stomata closure; subsequently, photosynthetic apparatus will be affected through declines in assimilation rate, transpiration, and intercellular CO_2 [30]. In our case, supplementing of PEG to the nutrient solution resulted in an early sharp reduction in all gas exchange parameters at 48 hours of drought stress (data not shown). However, the highest reduction in g_s (−35% and −62%) and A (−40% and −50%) was recorded on day eleven of PEG application, respectively, under both drought stress levels compared to the control plants (Figure 3). Accordingly, the successive decreasing in g_s in presence of high PEG level confirmed stomatal limitation for photosynthesis restriction activity [23]. Transpiration declining reflects an earlier plant response to reduction in the water potential around the root zone. In the hereby presented experiment, PEG-treated plants showed significant reduction in water loss and consistently in the transpiration rate (−27% and −53%), respectively, upon 2% and 4% of PEG (Figure 4). These results support the idea that the plants showed high ability to control the water fluxes through higher stomatal closure, which might be considered as the first line of defense against water loss and a protection mechanism to tissue dehydration [15]. Altogether, thyme plant showed a drought avoidance mechanism that involves a balance between water loss through the considerable reduction in g_s and in E values in PEG-treated plants (Figure 3) and water uptake by maintaining root growth development (Figure 1). These stomatal structural-functional changes might help the plants to adjust effectively to the unfavorable environmental conditions. Stomatal control and morphological root architecture are considered the key controlling factors for WUE under drought stress [31]. WUE expresses the relation between root water uptake ability and shoot water use. It is commonly recognized that higher WUE species have better adaptation to drought. The increase in WUE under water deficit was ascribed to the fact that either the biomass production is reduced less than water use [22] or the net CO_2 assimilation rate is being decreased less than transpiration rate [23, 30]. In this trial, PEG-treated plants maintained similar WUE to the nontreated ones (Figure 3). The maintenance of WUE that was associated with decreasing of osmotic potential of the nutrient solution upon adding PEG suggests that the decline in water absorption induced a partial photosynthetic damage as a response to drought stress. Alternatively, it was also possible that a partial inhibition of the photosynthetic function of CO_2 fixation, RUBP carboxylation, and inorganic phosphorus transformation occurred [32]. However, this negligible effect of drought stress on WUE suggested a strong relation between root water uptake and saving water use in thyme plant in condition of water scarcity [23]. Taken together, under drought stress condition, water consumption was reduced based on RWC value (Figure 2) altogether with plant fresh weight reduction (Figure 1) while the WUE (Figure 3) was kept as constitutive values suggesting that water shortage might improve plant WUE overall resulting in reducing production costs and resource use.

4.2.2. Essential Oils Composition. Responses of plant to drought stress in terms of volatile emissions are species specific and depend on the severity and duration of the stress period [7]. The presence of PEG increases plants osmotic pressure and causes induction of PEP carboxylase activity and oil biosynthesis [21]. The induction of this enzyme activity might be considered as an adaptive mechanism response to sustain photosynthetic carbon assimilation and maintain water status [21]. In our investigation, stress induced alteration in oil accumulation (Table 1). The emission of diisobutyl phthalate was enhanced under stress condition and summed up (+55.1%) under mild stress level. It has been suggested that under stress a higher oil glands density will occur, associated with a reduction in leaf area, and these conditions result in an elevated amount of oil accumulation [33]. Similarly, geraniol showed significant increasing in PEG-mild stressed plants compared to the control ones (28.4% versus 4.2%). Singh et al. [21] demonstrated that geraniol is the major constituent of the oil of lemongrass and the monoterpenoids are interconvertible under the catalysis of geraniol dehydrogenase [21]. Thus, we might suggest that this volatile plays a prominent protective role and might be, at least partially, one of the factors contributing to the modulation in oil yield under drought stress. It is known that the ratio of monoterpenoid phenolic compounds (e.g., thymol and carvacrol) plays a determinative role for cosmetic, culinary, and pharmaceutical products [34]. Thymol has been established as an essential oil component (58.1%) in *Thymus vulgaris* [35]. However, in this work thymol has not been reported as a main oil element under either control or mild stress, while a pronounced increasing upon high drought stress in measure of +4.4% was shown. The relevant increase (+31.7%) of carvacrol under high drought stress was consistent with Aziz et al. [4] and Said-Al Ahl and Hussein [36] who reported that the oil of *Thymus vulgaris* and oregano phenolic compound increased under stress condition. On the other hand, these findings were contrasting with those by Aziz et al. [4] and Bahreininejad et al. [37] who mentioned that the carvacrol content was reduced under both moderated and severe drought stress in *Thymus vulgaris* and *Thymus daenensis*. β-caryophyllene, as a sesquiterpene hydrocarbon, was negligible under control condition while it showed a significant increasing (+2.1%) at high drought stress. However, different literatures showed contradictory results of this component under water stress condition. Said-Al Ahl et al. [20] and Bahreininejad et al. [37] documented the reduction of this component upon drought stress in oregano and *Thymus daenensis* while Jordán et al. [7] and Said-Al Ahl and Hussein [36] demonstrated the rise of this component under moderate water stress and its reduction upon drastic stress condition in *Thymus hyemalis* and oregano plants. This volatile oil composition might be attributed to different plant genus, species, and accession as well as the trial conditions. The main outcome of oil analysis in this study illustrated that it is possible to orient the content of desired pharmacological components of thyme plant by manipulating different agricultural management techniques such as dynamic irrigation water management.

5. Conclusions

Thyme plants showed a water stress adaptive mechanism by restricting their growth and allocated more competitive carbohydrate to roots to promote the root absorption capacity. The plants were able to maintain the WUE and root to shoot ratio upon adding PEG which might indicate a strong relation between root water uptake and water use efficiency. In addition, the supplementing of PEG affected the water fluxes and caused higher stomatal closure and accordingly reduction in water loss through transpiration. The main oil components were influenced by drought stress. Some components increased significantly upon stress such as geraniol, carvacrol, and diisobutyl phthalate, while pseudophytol was reduced. Thus, desired pharmacological components of thyme might be oriented by manipulating water supply.

Competing Interests

The authors declare that they have no competing interests.

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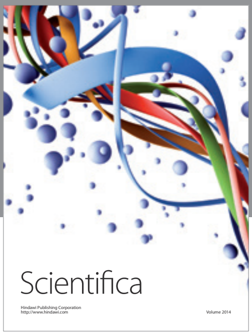
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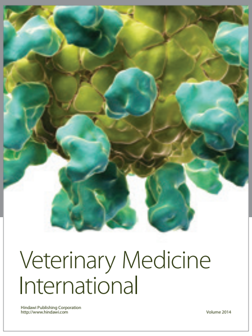
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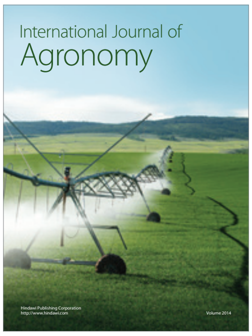
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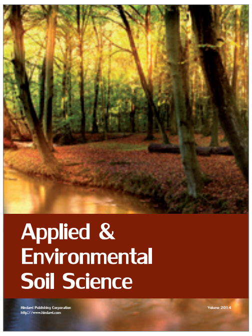
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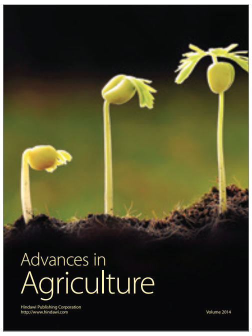
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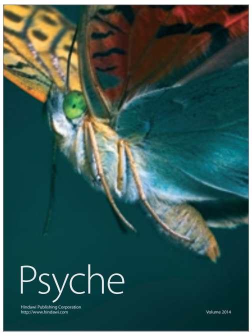
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