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EFFECT OF WATER SUPPLY ON GROWTH AND POLYPHENOLS OF LEMON BALM (*MELISSA OFFICINALIS* L.) AND THYME (*THYMUS VULGARIS* L.)

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A pot experiment was carried out with lemon balm (*Melissa officinalis* L.) and thyme (*Thymus vulgaris* L.). Different water supply was applied: 25%, 40% and 70% saturation of soil water capacity (SWC). Morphological traits, biomass and phenolic type active ingredients were investigated.

Among the two species, main differences were registered in biomass and TPC. Lower SWC resulted in reduced biomass production of lemon balm, while the applied stress treatments did not effect the biomass of thyme. In lemon balm, highest TPC contents were measured in control plants both in shoots and roots but in thyme, the shoots showed a significantly increased TPC at the 25% SWC conditions. Neither the content of total flavonoids nor that of the rosmarinic acid was affected by the treatments. The antioxidant capacity proved to be in tight connection with the TPC in both species (r = 0.766–0.883). The rosmarinic acid content of lemon balm plants contributed to the antioxidant capacity, as well (r = 0.679–0.869).

Keywords: Water capacity – drought stress – polyphenols – rosmarinic acid – antioxidant activity

INTRODUCTION

Information concerning the effect of water supply on medicinal and aromatic plants is still inadequate. Frequently, the optimum of dry matter production does not coincide with that of the accumulation of secondary compounds [6, 19]. Polyphenols, may act as defence molecules in biotic and abiotic stress conditions of the plants [8, 17, 28]. Their pharmacological effects include cardio-protective, gastrointestinal protection, anti-inflammatory, antimicrobial and antioxidant activity, etc. [11, 15]. In our experiment we compared two medicinal plants, lemon balm and garden thyme rich in polyphenols and, according to practical experiences, characterized by different water requirements.

Lemon balm (*Melissa officinalis* L.) is a perennial species cultivated all around the world. The most important biologically active components include the essential oil, the flavonoids, and the rosmarinic acid. The drug is justified as a sleep aid and a relief of mild symptoms of mental stress, and mild gastrointestinal complaints. Lemon balm is

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considered as a plant preferring good soil conditions, abundant nutrition and precipitation [25]. However, only sporadic data are available about the documented effects of water supply. Ozturk et al. [18] detected a significant loss of yield but an increase of essential oil accumulation if water deficit of the soil exceeded 25%. Shirzadi et al. [26], however, did not find any significant change in the biomass production of lemon balm due to drought stress. Unfortunately, in these publications the water dosage was not defined exactly. Farahany et al. [9] reported the highest essential oil content owing to the lowest water supply (20% field capacity) while the highest plant height and fresh mass was obtained at full water capacity. Investigation in soilless conditions carried out by Manukyan [16] who found a significant increase of polyphenols and rosmarinic acid under severe drought conditions (250 hPa soil moisture).

Garden thyme (*Thymus vulgaris* L.) is a commonly used culinary herb of Mediterranean origin. *Thymi herba* is reported to have strong antioxidant, antispasmodic, antitussive, expectorant, bactericide and adstringent properties [23]. Garden thyme contains a wide-range of phenolic type compounds: besides thymol and carvacrol, the main constituents of the essential oil, it accumulates flavonoids and rosmarinic acid, as well. In the agronomical practice, thyme is considered as a drought tolerant species. However, the majority of trials carried out under open field conditions ascertained that lower field capacity or longer irrigation intervals reduced plant height and herb yield while increased root/shoot ratio and essential oil content [1–4]. Accumulation of volatile phenolic compounds seems to be accelerated by warm and dry climatic conditions [2, 21], although contradictory findings have also been published [1]. Non-volatile phenolics are less studied. Khosh-Khui et al. [12] presented recently a slight decrease of total phenolics and antioxidant activity in thyme due to a ten day irrigation interval compared with a two days regime.

Our main goal was to detect how water supply influences the most important morphological traits, biomass and drug production as well as active ingredients of the experimental species. A special emphasis was put on the phenolic type components and their distribution in the aboveground and underground plant parts which have never been studied parallelly.

MATERIALS AND METHODS

Plant material and growth conditions

Three-month-old seedlings of lemon balm (*Melissa officinalis* L.) were used for the investigation, propagated from a population cultivated in Hungary commercially. Thyme (*Thymus vulgaris* L.) variety ‘Varico 3’ (seed supplier Mediseed, Switzerland) was raised from seeds at the experimental station of the department in greenhouse, then kept in open field in containers and used as 7-month-old young plants in the experiment.

Plants were grown under controlled conditions (Fitotron SGC120 growth chambers, Weiss Gallenkamp Ltd., Loughborough, Lencestershire, United Kingdom) in

Table 1
Environmental conditions applied in the growth chamber during the experiment (14-hour day and 10-hour night cycle)

Weeks	Day/Night temperature (°C)	Relative humidity (%)	Light intensity (μmol s^{-1} m^{-2})	
			Lemon balm	Thyme
1–3	14/10	65	195	195
3–8	20/14	65	195	260
8–9	22/16	55	195	260
9–19	25/16	55	195	260

2014. Programming of the chamber was based on a 14-hour day and a 10-hour night cycle. The details are summarized in Table 1. The growing medium above a gravel drainage (1000 g, cca. at 2 cm depth per pot) consisted of a 1:1 (v:v) mix of washed sand and perlite. Two plants were planted in each pot and 5 pots per treatment were used for both species. Each pot was installed to an equal 4500 g weight of medium. Plants were fertilized each week with 40 ml/pot of modified Hoagland solution which was increased from the 9th week of the experiment to 80 ml/pot. Treatment was initiated after 9 weeks of acclimatization following planting and harvest was carried out after 10 weeks of treatment. At the end of the experiment the most important morphological characteristics were recorded then all the plants were taken out from the pots and separated into shoot and root parts to determine the production and prepare samples for laboratory analysis.

Treatments

Three different levels of water supply were used to ensure 25% (S2 = severe drought), 40% (S1 = moderate drought) and 70% (C = control) saturation of soil water capacity (SWC). SWC was determined prior to the study using the gravimetric method [26]. Both SWC checking and irrigation were carried out three times per week, which was proven to be effective according to our previous experiences.

Measurements

Morphological characteristics

Plant height of both species was measured as the length of the longest shoot from the root neck to the tip of the shoot in both species. Parallell to this, root length was determined by measuring it from the root neck to the tip of the longest root. These measurements were carried out in 10 replications/treatment/species. The length of internodes and the diameter of stem were determined at the 3rd internode under the tip of the main shoots. These measurements were fulfilled in 15 replications.

At the end of the experiment the plant individuals were taken out of the pots and separated into shoot and root parts. The roots were cleaned and the plant material was measured to get the fresh mass. The plant parts were dried at room temperature till constant weight and the dry mass was registered. These measurements were carried out in 10 replications.

Relative water content (RWC)

The relative water content (RWC) was analysed from leaf samples originating from the third nodes under the tip of the shoots (in 6 replications/treatment/species). Following the determination of their fresh weight (FW) the samples were immersed in distilled water for 12 hours (for the estimation of the turgid weight – TW), then they were dried at 80 °C for 24 hours (for measuring the dry weight – DW). The RWC was determined by using the following formula [7]:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100 \ .$$

Total phenolic content (TPC)

For measuring the TPC and antioxidant activity, 1 g dried and powdered plant material was extracted by 100 mL boiling distilled water and was allowed to stand for 24 hours at room temperature. Then the extracts were filtered and stored in freezer until the measurements were taken place. The TPC was determined by the modified method of Singleton and Rossi [27]. Sample solution of 0.5 mL was introduced into a test tube and then 2.5 mL Folin–Ciocalteau's reagent (10 v/v%) was added. After 1 min of incubation 2 mL of sodium carbonate (0.7 M) was added. The absorbance of the resulting colour was measured at 760 nm with a Thermo Evolution 201 spectrophotometer after a 5 min incubation period in hot water (50 °C). Quantification was done with respect to the standard curve of gallic acid (0.3 M) and the TPC of the sample was expressed as mg of gallic acid equivalents (GAE) per g of dry weight of exctract. Blank was prepared to contain distilled water instead of extract. The measurements were carried out in triplicate.

Total flavonoid content

The flavonoid content was determined according to the method given in European Pharmacopoeia VIII [20] for *'Equiseti herba'* using half of the amounts of materials described there. Shortly, 0.4 g dried and powdered plant material was extracted by boiling in 1 mL hexametilentetramine, 20 mL acetone and 2 mL HCl for 30 minutes, then filtered. Afterwards the extraction was repeated twice by 20 mL acetone by boiling the solution for 10 minutes. The extract was filtered and diluted with aceton to

100 mL volume. Twenty mL of extract was mixed with 20 mL water and 45 mL ethyl-acetate and shaken thoroughly. After incubation for 30 minutes the absorbance was measured at 760 nm in the spectrophotometer mentioned above. The total flavonid content of the samples was expressed in isoquercitroside (QE) per dry weight of plant material. Blank was prepared from acetic acid and methanol. The measurements were carried out in triplicate.

Rosmarinic acid content

For the determination of rosmarinic acid (RA) content 500 g powdered dry plant material was suspended in 45 mL methanol. The suspension was heated for 30 minutes in water bath, cooled after that and filtered (by 45 µm filter) into a 100 mL flask. The filtrate was completed by methanol to 50.0 mL volume. RA content was determined by HPLC method in triplicate. The Waters HPLC system consisted of a 1525 binary pump with a 717plus autosampler, a Jetstream column thermostate and a 2998 PDA detector, controlled by Empower2 software. A Kinetex C-18 column was used, 100 mm L 4,6 mm i.d., 2,6 µm particle size. All solvents were HPLC grade. For the elution, 1 : 19 : 80 phosphoric acid : acetonitril : water (mobile phase A) and 1 : 40 : 59 phosphoric acid : methanol : acetonitrile (mobile phase B) were used as solvents at a flow rate of 1 ml min^{-1} [20]. The gradient program started at 100% A and after solvent B was increased linearly and reached 35% in 10 min, then 100% in 2 min. Finally, 100% A was reached at 2 min. 8 min post-time was set for the equilibration of the initial solvent composition. The column temperature was maintained at 35 °C and the injection volume of 5 µL was used in all experiments.

Antioxidant activity

The FRAP assay was performed according to the Benzie and Strain [5] procedure with slight modifications. One g dried and powdered plant material was extracted by 100 mL hot distilled water and was allowed to stand for 24 h. Then the extracts were filtered and stored in freezer until the experiments took place. FRAP reagent was prepared freshly to contain sodium acetate buffer (pH 3.6), TPTZ (2,4,6-tripiridil-s-triazin) in HCl and $FeCl_3 \cdot 6H_2O$ solution (20 mmol/L), in proportion 10 : 1 : 1 (v/v/v), respectively. Ten µL of test sample was added to 1.5 mL of acting FRAP reagent and 40 µL distilled water and absorbance was recorded at 593 nm after 5 minutes using the spectrophotometer above. Blank contained distilled water instead of extract. FRAP values of samples were calculated from standard curve equation and expressed as mg ascorbinic acid equivalent (AAE)/g of dry extract.

Statistical analysis

The results were analysed with the IBM SPSS Statistics 22 software. Means, standard deviations, coefficient of variation, and one-way ANOVA was applied. Normality of the residuals was proved according the Kolmogorov–Smirnov method. Homogeneity of variances was tested by Levene's method. Treatments were separated by Games–Howell's or Tukey's post-hoc tests, depending on whether homogeneity assumption was violated or not. For evaluating the connection of data Pearson correlations were performed.

RESULTS

The main morphological characteristics showed similar results in the two experimental species respecting the water supply. The length of internodes was definitely shortened in the case of reduced SWC. However, the treatments had no significant effects either on shoot and root length or on stem diameter of lemon balm and thyme (Tables 2–3).

The biomass of both species decreased in the case of lower water supply (Tables 2–3). In lemon balm the fresh mass of the S2 plants reached only 72% of that in the control pots. However, the reduction was not significant until 40% SWC. Poor water supply also decreased the root mass without reaching the significance level. In thyme, decrease of fresh shoot mass reached more than 16% and that of the fresh root mass was 39% due to the lowest SWC (Table 3). Dry masses followed the same tendencies detected in case of fresh mass in both species.

In lemon balm the relative water content of the leaves varied between 86.1 and 87.7% with a very low standard deviation (coefficient of variation = 1.8–3.8%), (Table 2). In thyme, the values are somewhat higher (88.9–91.9%), but even here no effect of the treatments could be registered and standard deviation is low (coefficient of variation = 1.1–3.8%), (Table 3).

In the shoots of lemon balm TPC values between 391 and 478 mg GSE/g DW were detected. The levels in the roots represent only 61–75% of that of the shoots (Table 2). Concerning the shoots, the significantly lowest concentration was measured at the S1 treatment reflecting no exact tendency among the treatments. In the roots, accumulation is decreasing: in S2 plants it is only 82% of the value obtained at the C plants. In thyme, generally a higher accumulation rate of total phenolics could be measured than in lemon balm (Table 3) and they were affected significantly by water deficiency. We have found that the TPC in the shoots increased by decreasing SWC, however, a quite opposite tendency was established in the roots: highest contents were achieved at control plants.

In lemon balm, the RA contents of the shoots of each sample exceed the 1% minimum requirement of the Pharmacopoea Europaea VIII [24], (Table 2). The RA content of the roots is also considerable, reaching 53–66% of that of the shoots. However, no significant changes in these values could be detected as result of the treatments.

Table 2
Effects of water supply on the parameters in lemon balm (mean ± Standard deviation)

Parameter	Treatments (soil water capacity = SWC)		
	70%	40%	25%
Stem height (cm)	22.5^{a} ± 2.65	23.5^{a} ± 2.38	26.25^{a} ± 6.13
Root length (cm)	37.5^{a} ± 2.51	38.6^{a} ± 8.96	37.6^{a} ± 5.19
Internode length (cm)	1.80^{a} ± 0.14	1.48^{b} ± 0.21	1.38^{b} ± 0.05
Stem diameter (cm)	1.58^{a} ± 0.21	1.54^{a} ± 0.22	1.70^{a} ± 0.12
Fresh mass of shoots (g)	40.3^{a} ± 1.1	33.9^{ab} ± 8.8	29.0^{b} ± 2.9
Dry mass of shoots (g)	8.6^{ab} ± 0.5	9.2^{a} ± 0.50	7.7^{b} ± 1.0
Fresh mass of roots (g)	66.2^{a} ± 5.1	46.7^{a} ± 18.0	47.9^{a} ± 14.4
Dry mass of roots (g)	11.5^{a} ± 1.1	8.1^{a} ± 2.9	8.9^{a} ± 2.8
Relative water content of shoots (%)	87.7^{a} ± 1.6	86.9^{a} ± 2.7	86.1^{a} ± 3.3
Total phenolics content, shoots (mg GAE/g DW)	484.43^{a} ± 72.67	390.72^{b} ± 56.53	477.83^{a} ± 38.34
Total phenolics content, roots (mg GAE/g DW)	355.85^{a} ± 38.87	295.24^{b} ± 20.55	293.23^{b} ± 23.37
Rosmarinic acid content, shoots (%)	2.79^{a} ± 0.28	2.57^{a} ± 0.15	2.64^{a} ± 0.16
Rosmarinic acid content, roots (%)	1.79^{a} ± 0.39	1.36^{a} ± 0.06	1.39^{a} ± 0.15
Total flavonoids content, shoots (mg QE/g DW)	0.760^{a} ± 0.060	0.676^{a} ± 0.127	0.799^{a} ± 0.222
Total flavonoids content, roots (mg QE/g DW)	0.100^{a} ± 0.064	0.028^{a} ± 0.006	0.095^{a} ± 0.046
Antioxidant capacity, shoots (mg AAE/g DW)	342.40^{a} ± 84.08	220.73^{b} ± 36.88	323.05^{a} ± 62.71
Antioxidant capacity, roots (mg AAE/g DW)	269.08^{a} ± 32.15	205.41^{b} ± 24.97	213.37^{b} ± 24.97

Different letters represent significant differences in the rows.

The findings were rather similar for thyme, as well (Table 3). The roots contained 0.977–1.191% RA which takes 50–78% of that of the shoots. The values, did not reflect any effect of the applied soil water levels, similarly to lemon balm.

Total flavonoid content of the shoots of lemon balm varied between 0.676 and 0.799 mg QE/g DW while in thyme the values were 1.102–1.150 mg QE/g DW (Tables 2–3). On contrary to the above-mentioned TPC and RA content, the concentrations of flavonoids in the roots are relatively low, reaching only 4–13% of the levels of the shoots in the case of lemon balm and 0–7% of that in thyme. The experimental treatments did not affect the total flavonoid content in either of the species.

Table 3
Effects of water supply on the parameters in thyme **(**mean ± Standard deviation**)**

Parameter	Treatments (soil water capacity = SWC)		
	70%	40%	25%
Stem height (cm)	$26.5^{a} \pm 5.8$	$27.6^{a} \pm 3.3$	$29.2^{a} \pm 4.2$
Root length (cm)	$23.8^{a} \pm 4.6$	$25.0^{a} \pm 2.8$	$20.5^{a} \pm 1.0$
Internode length (cm)	$1.50^{a} \pm 0.35$	$1.03^{b} \pm 0.15$	$1.30^{a} \pm 0.26$
Stem diameter (cm)	$0.58^{a} \pm 0.14$	$0.54^{a} \pm 0.23$	$0.45^{a} \pm 0.10$
Fresh mass of shoots (g)	$20.1^{a} \pm 5.8$	$17.4^{a} \pm 1.5$	$17.2^{a} \pm 3.4$
Dry mass of shoots (g)	$9.3^{a} \pm 2.9$	$7.7^{a} \pm 0.5$	$7.8^{a} \pm 1.4$
Fresh mass of roots (g)	$14.6^{a} \pm 11.5$	$15.7^{a} \pm 3.57$	$10.5^{a} \pm 4.05$
Dry mass of roots (g)	$3.72^{a} \pm 2.25$	$3.23^{a} \pm 0.87$	$3.5^{a} \pm 0.77$
Relative water content, shoots (%)	$91.1^{a} \pm 1.89$	$88.8^{a} \pm 3.4$	$91.9^{a} \pm 1.1$
Total phenolics content, shoots (mg GAE/g DW)	$695.34^{b} \pm 99.15$	$736.08^{b} \pm 50.24$	$904.37^{a} \pm 158.75$
Total phenolics content, roots (mg GAE/g DW)	$840.13^{a} \pm 48.35$	$672.21^{b} \pm 77.84$	$688.88^{b} \pm 108.69$
Rosmarinic acid content, shoots (%)	$1.942^{a} \pm 0.369$	$1.816^{a} \pm 0.416$	$1.938^{a} \pm 0.075$
Rosmarinic acid content, roots (%)	$1.052^{a} \pm 0.144$	$1.191^{a} \pm 0.388$	$0.977^{a} \pm 0.137$
Total flavonoid content, shoots (mg QE/g DW)	$1.042^{a} \pm 0.226$	$1.150^{a} \pm 0.175$	$1.102^{a} \pm 0.096$
Total flavonoid content, roots (mg QE/g DW)	0.000	$0.045^{a} \pm 0.077$	$0.080^{a} \pm 0.032$
Antioxidant capacity, shoots (mg AAE/g DW)	$508.70^{a} \pm 72.80$	$504.89^{a} \pm 81.92$	$607.51^{a} \pm 166.94$
Antioxidant capacity, roots (mg AAE/g DW)	$604.80^{a} \pm 93.63$	$540.64^{ab} \pm 58.60$	$482.40^{b} \pm 138.04$

Different letters represent significant differences in the rows.

In lemon balm the antioxidant activity both of the shoots and the roots showed the lowest values at the S1 treatment. In the shoots, this level was exceeded in both the wetter and the drier soils (Table 2). In the roots the increased antioxidant capacity however, was associated with a better water supply. Antioxidant capacity of thyme – using the same method – was 2–3-fold higher than that of lemon balm. The highest value (607.51 mg AAE/g DW) was found in the shoots at the S2 treatment (Table 3). In the case of the roots, the antioxidant capacity was comparable with that of the shoots and a firm tendency could be established connected to water supply: lower SWC resulted in significantly lower antioxidant capacity.

Table 4
Connections between antioxidant capacity (AC) and different phenolic compounds

Content of	AC (mg AAE/g DW) lemon balm				AC (mg AAE/g DW) thyme			
	shoot		root		shoot		root	
	r	p	r	p	r	p	r	p
Total phenolics (mg GAE/g DW)	0.883*	0.000	0.808*	0.000	0.766*	0.000	0.827*	0.000
Total flavonoids (mg QE/g DW)	0.488	0.183	0.328	0.389	–0.010	0.980	–0.249	0.519
Rosmarinic acid (%)	0.679	0.044	0.869*	0.002	0.425	0.254	0.268	0.485

r – Pearson correlation coefficient; p – significance level; *significant at $p < 0.01$.

DISCUSSION

Changes in the morphological features of the plant species studied were only tendency-like, except the length of internodia. Former experiences in open field showed that drought stress reduced the length of internodia and diameter of stem of lemon balm [9] and plant height of thyme [1, 3]. Our findings show, however, that under the circumstances of this experiment both species were able to tolerate the water deficiency in the ten-week period of stress treatments without significant changes concerning morphological features.

Plant biomass of both species showed a decreasing tendency when SWC was reduced, however, it was only significant in lemon balm shoots. This is in accordance with the fact that it is known as a more water demanding species in the practice. While the loss of biomass due to lower SWC in lemon balm is comparable with former references [9, 16], the lack of significant changes in thyme may contradict to some previous data [1, 4]. Unfortunately, each publication represents different conditions, thus hardly comparable with our data.

The elevated level of total phenolics by 30% in the shoots of thyme at the S2 treatment might reflect a better adaptation to drought conditions compared to lemon balm. This is in accordance with several former literature data in other species [8, 17] but contradicts to a recent reference on thyme which described a decreasing tendency of TPC due to longer irrigation intervals [12]. According to our knowledge, the results achieved are absolutely new concerning the TPC of the roots in both species. It seems to be important that the underground parts of thyme contain almost the same concentrations of phenolics as the aboveground shoots. It has been shown in cereals that both shoots and roots react specifically to abiotic stresses [14]. Even more interesting is that there is an opposite tendency of changes in the two plant organs due to lower water supply. It needs further research to clear up the connection between the biosynthetic mechanisms of the two plant parts and/or the possible transport processes.

Although RA could be detected in each plant sample of both experimental species, the applied stress treatments could not induce any change in its level. Consequently,

the findings of Manukyan [16] could not be ascertained under the circumstances of our pot experiment.

It was found that the flavonoids are present also in the roots of both species although at a magnitude lower level compared to the shoots. Fini et al. [10] noticed that flavonoids may play important role as secondary scavengers and antioxidants in certain stress situations; it was not justified by our results for lemon balm and thyme. In former references we have not found any data on the accumulation of flavonoids as a consequence of water supply in these target species.

The antioxidant capacity of the samples proved to be in tight ($p < 0.01$) connection with the TPC in both species and in both the shoots and roots (Table 4). It is in contradiction with the results of Manukyan [16] for lemon balm but ascertains several data on other species [23, 24]. Connection of FRAP antioxidant capacity and TPC was also described for *Thymus vulgaris* previously, although both characteristics of the herb decreased as an effect of low water supply [12]. The RA content of lemon balm plants contributed to the antioxidant capacity, as well while no correlation between these latter two parameters were found in thyme (Table 4).

Among the two species, main differences were registered in biomass and TPC. Lower SWC resulted in reduced biomass production of lemon balm, while the applied stress treatments did not effect the biomass of thyme. In lemon balm, highest TPC contents were measured in C plants both in shoots and roots but in thyme, the shoots showed a significantly increased TPC at the S2 conditions.

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