Final report on the project "Background and regularities of accumulation of phenolic compounds in medicinal plants" (NN 108633)

<u>Abbreviations:</u>
TPC total phenolic content
TFC total flavonoid content
THC total hydroxycinnamic acid content
RA rosmarinic acid
EO essential oil
AC antioxidant capacity
SWC saturation of soil water capacity
DM dry weight
MAP medicinal and aromatic plants

The project entitled above has been running from 1st January 2014 till 31 March 2017. Principally we followed the planned activities as described in the original work plan. We used two model species: lemon balm (*Melissa officinalis* L.) and garden thyme (*Thymus vulgaris* L.) to compare their reactions to the drought stress treatments. The experiments were carried out each in climatic chambers, in covered place as pot experiment during the vegetation year and also as parallel open field trials in two habitats. Water supply was regulated in each case as determined by the saturation of soil water capacity. In each experiment several morphological parameters (plant height, plant diameter, leaf size, number of branches, etc.), biomass and drug yield, secondary compounds (as total phenolic content, total flavonoid content, hydroxyl-cynnamic-acid content, rosmarinic acid content, essential oil content and main components) furthermore the antioxidant capacity of the samples were determined. The level of the stress has been controlled also by physiological and other measurements such as relative water and dry matter content of the tissues, water potential and chlorophyll content of the leaves.
In the followings, the main results and conclusions have been summarized according to the main goals of the research as it has been described in the *Research plan* at the time of application.

1. ***Chemical variability of the species***

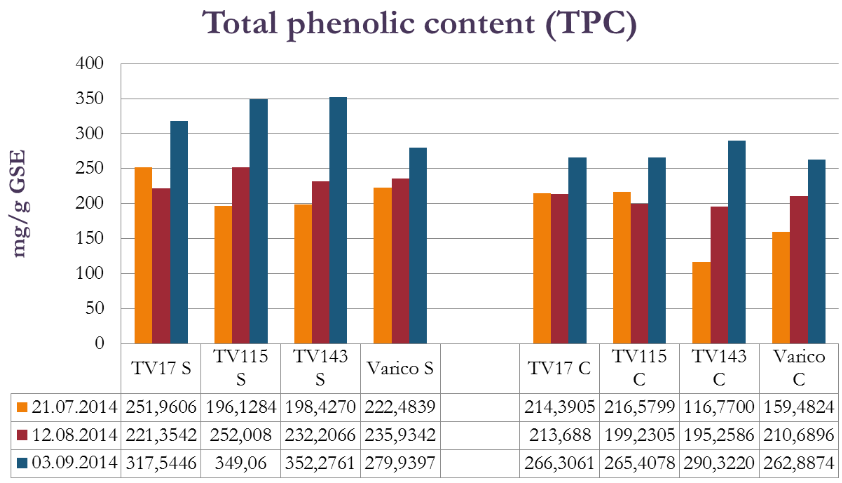
In the first year we checked the intraspecific chemical variability -focusing on phenolics- of both species. 5 commercially available genotypes of *Melissa officinalis* were chosen (Szabó et al., 2016). In case of thyme, four varieties ('Varico 3', 'Sloneczko', 'Standard Winter', 'French Summer') and three chemotypes, -determined formerly by their main EO compounds- from our collection (cv. thymol: TV17; cv. geraniol: TV115; cv. terpineol: TV143) have been used. No data were available on the phenolic type compounds of these intraspecific accessions. They have been investigated in a pot experiment in 2014 and in the parallel growing plave experiment (Soroksár-Poznan) during three years.
In lemon balm significant differences could be detected among the TFC, THC and FRAP antioxidant capacity of the genotypes, while there were no significant differences among genotypes comparing their TPC as well as RA content. 'Lorelei' and 'Lemona' accumulated the highest levels of TFC, while the highest TPC was measured in variety 'Gold Leaf'. The content of RA varied between 2.43 ('Lemona') and 3.01% DW ('Lorelei'). The THC was the highest in the samples of variety 'Gold Leaf' (8.07%). 'Lemona' accumulated by far the highest essential oil level (0.298 mL/100g DM.). The qualitative spectrum of the main volatile compounds however, was similar in each tested material and only small quantitative differences in the ratios of the main compounds were detected. Besides, the accumulation of the most important phenolic compound, RA showed high variability even within cultivars in some cases.

It could be established that the non-volatile, phenolic type active ingredients displayed low variability among the lemon balm cultivars.
Concerning the chemical variability of different thyme accessions, no significant differences among the tested varieties had been found in TPC (between 200.78 and 253.32 mg/g GSE), similarly to lemon balm. Differences manifested themselves however, each in case of TFC, AC, EO and RA content. TFC and EO content was highest in 'Varico' (1.039% and 3.97 %, respectively), AC exhibited highest value in 'French Summer' (238.917 mg/g ASE). In the level of RA accumulation, differences among varieties up to 22% were registered. We have found that the level of RA was irrespective of the EO chemotype. Furthermore, it was found that growth conditions and/or location may influence also the differences among the genotypes (see later).
To get more knowledge on the chemical properties of our species, -although it has not been planned before,- in the first year we took samples also from the roots of both species. We determined their phenolic contents (TPC, TFC and RA content) which has not been investigated before and no literature data were available about them (Németh et al., 2015 and 2017). TPC of the roots of thyme was at the same level than it is in the shoots. Lemon balm exhibited a different accumulation in phytotron and in semi-controlled conditions: while in the former one the TPC of the roots was similar (290-360 mg GAE$\cdot g^{-1}$ DM) to that of the shoots, it was much lower in the pots (60-70 mg GAE$\cdot g^{-1}$ DM).
For RA similarly, almost the same level was detected in the shoots and in the roots in the phytotron but a magnitude lower levels were measured in the pot experiment and it was characteristic for both species. Presumably, the age of the plants may also play a role in these contents as in the climatic chamber younger plants were used.
There was a practically very low level (below 0.1mg QE/g DM) of flavonoids in the roots of both species not comparable with that of the shoots.

2. *Changes in phenolic compounds due to water supply*

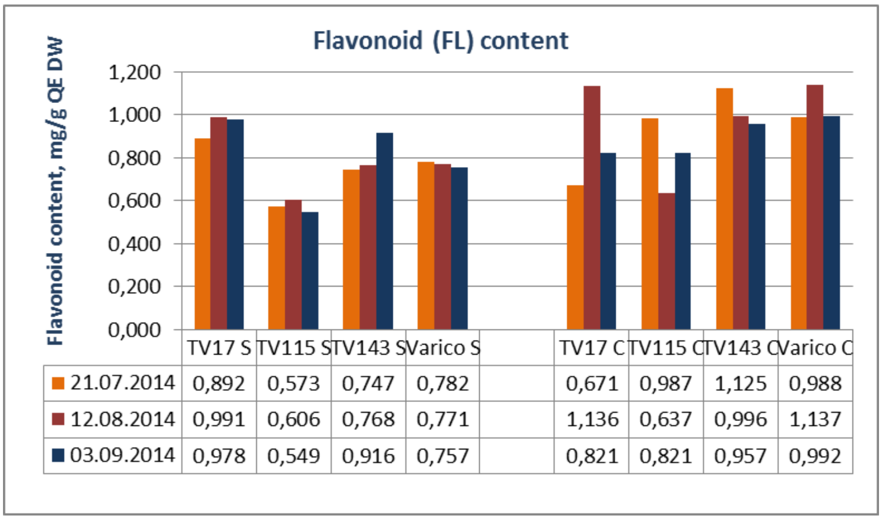
In the first experiment we applied three regimes of water supply: severe stress conditions (SWC 25%), medium water supply (SWC 40%) and control (SWC 70%). This trial has been installed in phytotron and the growth medium was a sand/perlite one. Lack of water resulted in increasing values of TPC and AC in thyme but no significant change was registered in lemon balm. TFC and RA was not affected by the treatments in either of the species (Németh et al., 2015).
Based on the experiences, the trial was followed by an extended study in form of a pot experiment at an open field plot (covered by plastic roof, semi-controlled conditions), using soil medium (Florasca Bio B) and two treatments of water supply: "stress" (SWC 40%) and control (SWC 70%) conditions during three months (Szabó et al., 2015). Sampling was taken three times in order to follow also the tendencies of mutual changes.
The TPC of the lemon balm varieties decreased in general, due to the lower water supply. At the same time it could be observed that the varieties did not exhibit a uniform response to the treatments. Similar finding was observable also for TFC which increased only in 'Gold Leaf' and 'Soroksár' being not significantly affected in the other three genotypes. THC presented an increased accumulation in cv. 'Lorelei' while three varieties reacted with lower accumulation, and no significant effect was registered for 'Quedlinburger Niederliegende'. The content of RA interestingly, showed only weak interaction with variety as lower accumulation level was measured in each sample in consequence of lower water supply. It could be concluded that in lemon balm the level of total phenoloids and that of RA most likely do not participate as defence compounds as response to low soil water content.
In the same experiment mentioned above, the other species, garden thyme exhibited more characteristic changes in phenoloid accumulation due to the restricted water supply. TPC increased by 6-32% in the „stressed" plants with some differences among accessions. It should

be mentioned that this difference was detectable already three weeks after starting of the treatments (**Figure 1**.). Regarding TFC of thyme shoots, in general, lower concentrations were detected under stress (S) conditions. However, during the experimental period the observed tendencies of accumulation seem to be contradictious (**Figure 2**.). RA, the compound in focus of our project showed increased concentrations in the plants grown at lower soil water content (**Figure 3**.). This increase reached 93% (in accession’TV17’) but as lowest it was also 40% (variety ’Varico’). For each measured parameters it could be observed that although the rate of the change between control and stressed plants reflected genotype-dependence, the direction of these changes was the same in each accession. This is a clear difference compared to lemon balm, where in most cases even the direction of the changes was not uniform.



	TV17 S	TV115 S	TV143 S	Varico S		TV17 C	TV115 C	TV143 C	Varico C
21.07.2014	251,9606	196,1284	198,4270	222,4839		214,3905	216,5799	116,7700	159,4824
12.08.2014	221,3542	252,008	232,2066	235,9342		213,688	199,2305	195,2586	210,6896
03.09.2014	317,5446	349,06	352,2761	279,9397		266,3061	265,4078	290,3220	262,8874

Figure 1. Effect of drought stress on total phenoloid content of thyme chemotypes (mg/g GSE)



	TV17 S	TV115 S	TV143 S	Varico S		TV17 C	TV115 C	TV143 C	Varico C
21.07.2014	0,892	0,573	0,747	0,782		0,671	0,987	1,125	0,988
12.08.2014	0,991	0,606	0,768	0,771		1,136	0,637	0,996	1,137
03.09.2014	0,978	0,549	0,916	0,757		0,821	0,821	0,957	0,992

Figure 2 Effect of drought stress on flavonoid content of different thyme chemotypes (mg/g QE)

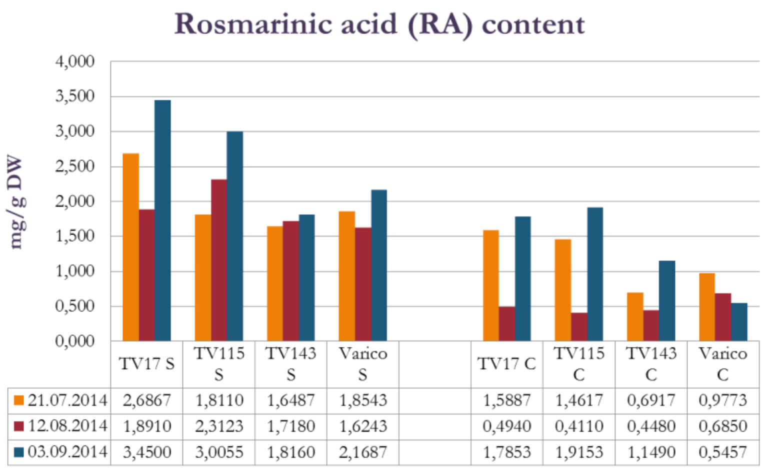


Figure 3. Effect of drought stress on rosmarinic acid content of thyme (% DW)

From the second year, instead of a standard water supply, changes in SWC during the experimental period were also included into the observations and the treatments were installed in order to study also **the effects of timing and duration of stress condition** on the tolerance responses of the target species.
In these trials treatment S1 (40% SWC) presented a continuous water shortage while in treatment S2 (40% SWC) the stress situation was started only three weeks later than in S1, thus it was shorter than that one. An addition, a S3 treatment has also been installed which represented a situation when water supply is gradually decreasing during the study period assuring –hypothetically- better possibility to the plants being acclimatized. As control 70% SWC was applied, similarly to the previous experiments. The experiments have been carried out both under controlled conditions in climatic chambers and under semi-controlled conditions under plastic roof in pots. In the latter situation after the experiences in phytotron chambers the gradually decreasing water level as treatment –which proved to be very sensitive to temperature and air humidity changes and difficult to regulate exactly- has not been applied.
In the last year (2016) this kind of study has been enriched with a further aspect: after the described treatments a three weeks long "regeneration" period was installed by keeping the water supply at 70% SWC for each plant. In harmony with the research plan, by this method we wanted to study the regeneration behaviour of the plants suffering under different levels of drought stress previously.
In case of lemon balm, continuous low level of soil water resulted in no significant changes in TPC, TFC and RA content (**Table 1**), (Radácsi et al., 2016). Plants in the treatment with gradually decreasing soil water content also produced almost the same results as *control* plants for all measured parameters except TFC. Plants in treatment S2- where the water supply suddenly decreased at the end of the experiment – accumulated less TPC and their antioxidant capacity was much lower as well. The results indicated that longer term drought and continuously decreasing water supply do not induce large changes in the accumulation of phenoloids as possible defence compounds of lemon balm. It is in coincidence with the data of former experiments of this project presented above. The sudden loss of water content of the soil however indicated another situation which had to be cleared up by the further study.

Table 1. Effect of different water supply on the phytochemical characteristics of *Melissa officinalis* (mean ± standard deviation)

Parameters measured	Control	Gradually decreasing water	Short term water deficiency	Long term water deficiency

		supply		
TPC mg GAE g^{-1} DM	146.45 a ± 9.19	149.71 a ± 17.06	105. 93 b ± 9.60	178.10 a ± 44.17
TFC mg QE g^{-1} DM	1.54 a ± 0.09	1.33 b ± 0.02	1.22 b ± 0.01	1.47 a ± 0.01
RA % DM	3.12 a ± 0.26	3.59 a ± 0.16	3.83 a ± 0.41	3.17 a ± 0.36
AC mg AAE g^{-1} DM	131.46 b ± 9.48	138.96 b ± 17.18	66.14 c ± 13.51	201.55 a ± 27.39

Different letters in rows represent significantly different values

Therefore the work has been continued by the experiments under semi-controlled conditions in 2015 and 2016 with similar treatments as mentioned before (Németh et al., submitted, 2017). In the samples of lemon balm we detected an elevated level of TPC both in the longer stress and in the shorter stress treatments but only in the second year. In 2015 the concentration was highest in the control plants (**Table 2**.). AC showed a tight connection with the phenolic values, and thus, findings are similar to that: in the first year the capacity was highest in the control while in the second year both stressed variants exhibited practically equal values, by 18% higher than that of the control. RA showed a significantly higher concentration in the continuously stressed plants in 2015 but there were no differences among the samples in 2016.
After the three-week regeneration period applied in the second year, each parameter increased significantly compared to the values measured immediately after the stress period. The increase was 22-64% for TPC, 49-67% for RA and 10-36% for AC. This time there were no significant differences registered any more among the treatments. As the mentioned data do not reflect any exact tendency of phenolic accumulation due to different water supply, it can be established that they are principally in harmony with the results of the former experiments. Thus, it seems to be most likely that phenolic compounds might play a secondary role in drought tolerance of lemon balm. Based on this, the elevated values of active ingredients after the three-week regeneration period that could be detected in each treatment might be in connection with other factors than water supply, e.g. age of the plants, temperature, etc. This aspect would be worth of further research.

Table 2. Changes in active constituents of the drug in the experimental years at different sampling times

Parameters measured	2015			2016					
	End of stress period			End of stress period			After regeneration		
	control	short term stress	long term stress	control	short term stress	long term stress	control	short term stress	long term stress
Total polyphenols (mg GAE·g^{-1} DM)	327.0a ±13.14	295.1b ±25.54	308.0 ab ±29.42	153.4 Bb ±13.76	208.7 Ba ±33.50	191.2 Ba ±13.33	252.5 Aa ±20.74	255.6 Aa ±41.23	233.8 Aa ±26.92
Rosmarinic acid content (% DM)	3.509b ±0.192	2.911c ±0.416	4.425 a ±0.646	2.815 Aa ±1.363	3.351 Ba ±0,426	2.777 Ba ±0.428	4.589 Aa ±0.874	4.999 Aa ±0.041	4.647 Aa ±0.749
Antioxidant capacity (mg AAE·g^{-1} DM)	257.9a ±25.59	175.7b ±45.36	227.9 a ±41.45	226.0 Bb ±16.23	266.8 Ba ±27.11	266.7 Ba ±31.24	308.5 Aa ±48.28	302.4 Aa ±17.61	293.0 Aa ±18.53

Capital letters indicate sign diff ($p>0.05$) between the same treatments in the two sampling times
Lower case letters indicate sign diff ($p>0.05$) among treatments at one sampling time

In the same situations, -in experiments with drought stress applied for different duration and timing- we experienced to some extent different results in thyme, compared to lemon balm. Statistically approved differences have been found for each chemical characteristics measured (**Table 3**). TPC, TFC and AC content increased as the drought became sever, plants under long term drought conditions represented the highest values (TPC:283.40±16.49mg/gGSE; TFC: 1.72 ± 0.02 mg/g QE; AC: 275.44±2.66 mg/g ASE; which are by 48, 16 and 45% higher than the values in the control samples, respectively. Rosmarinic acid which is the leading compound of the phenolic fraction in thyme also increased as availability of water became lower. RA reacted directly to the sudden water reduction, it exhibited the highest value (4.61±0.58%) in short term stress treatment. Nevertheless, the concentration in long term treatment is by 150% higher than that of the control plants, too.

Table 3. Summary of the results of different water supply treatments applied in growth chambers on *Thymus vulgaris* L. 'Varico 3' (Budapest, 2015)

Parameters measured	Treatments			
	control	gradually decreasing water supply	short term water deficiency	long term water deficiency
TPC mg GAE g^{-1} DM	191.68a ± 3.61	233.18a ± 8.36	254.52a ± 28.26	283.40b ± 16.49
TFC mg QE g^{-1} DM	1.48a ± 0.06	1.65a ±0.02	1.37a ± 0.01	1.72b ± 0.02
RA % DM	1.27a ± 0.05	2.99a ± 0.15	4.61b ± 0.58	3.185a ± 0.04
AC mg AAE g^{-1} DM	190.22a ± 7.33	210.01a ± 23.96	252.73a ± 54.27	275.44b ± 2.66

Different letters in rows represent significantly different values ($p<0.05$)

In the pot experiment under semi-controlled conditions the majority of these results in thyme could be ascertained. TPC was by 11% (2015) and 28% (2016) higher in the drought stressed plants than in the control ones. The effect of the duration of the treatments was well demonstrated, too. The three weeks stress treatment seemed not to be not long enough- it could not induce significant elevation in the phenolic content in either of the years. Besides, it was also observed that during the three weeks regeneration phase the differences caused by the longer term drought could not disappear. The weak regeneration capacity of thyme plants had been manifested not only in the secondary compounds but also in the growth parameters (see in chapter 4). These data are in contradiction with the ones gained in lemon balm which exhibited a more quick regeneration capacity.
As for the other phenoloid parameters, production of flavonoids has not been induced in these pot experiments, the TFC was highest in case of the control samples.
The accumulation dynamics of the RA however, ascertained the data obtained in the former phytotron experiment. The drought accelerated the accumulation of RA. Surprisingly, higher values have been detected in the shorter stress treatment than in the plants treated already for six weeks by water shortage. In conclusion, the increase of RA level might be a sudden reaction of thyme to water deficit. After the three weeks regeneration phase of uniform water supply (at 70% SWC) RA decreased in each plant again:

RA content of thyme shoots (2016, Budapest)

Treatments	End of stress	End of regeneration
control	2,258	2,116
short term	3,495	2,93
long term	2,872	2,671

It may be important to mention, that the changes of root phenoloids had been determined, too. (Németh et al., 2015 és 2017). To the contrary of the shoots, lemon balm reacted by significant elevation of TPC, however in RA content a significant decrease was measured.
In thyme, these changes were the opposite: TPC decreased while RA content increased in the roots. If there these changes are the results of de novo synthesis and/or translocation mechanisms between root and shoot are involved, would need further studies.

3. *Plant development and growing location as influencing factors in phenolic accumulation*

Goals of the project included also studying the connection between possible other biotic and abiotic factors and the drought stress response of the target species. One of these factors is the intraspecific diversity and characteristic reactions of the different accessions, which has been mentioned shortly in Chapter 2. The role of plant development proved to be an interesting and not negligible factor, too. The effect of ontogenetic phases and that of the growing habitat has been investigated during three years at two locations (Budapest and Poznań).
The **TPC** of lemon balm **decreased** during the progress of phenological stages, although the tendencies are **not without fluctuations** (**Table 4**). As for the effect of growing location, no clear indications could be stated. Accumulation of **total flavonoids** was also depending on the harvesting phase: during the vegetation year a continuous **decrease** was detected (**Table 5**). The environmental differences between the two growing locations modified the concentrations, usually the values were by 10-20% higher in Poznań. In the tendencies, no special reaction of the variety could be established.

Table 4. Total phenolic content (TPC mg GSE/g dw. ±sd.) of lemon balm cultivars in four phenological phases in two locations (2015)

Location	Cultivar	Phenophase			
		vegetative	budding	flowering	after flowering
		TPC mg GSE/g DM ±sd.			
Soroksár	'Gold Leaf'	226.0cd±30.53	249.1ef ±15.02	-	-
	'Lemona'	303.3d ±29.84	394.8a ±33.86	222.1cd ±11.48	160.0cd ±12.80
	'Lorelei'	-	352.6bc ±18.52	163.7d ±9.40	336.2a ±16.96
	‚Quedlinburger Niederliegende'	334.0bcd ±31.37	360.8ab ±38.15	165.4d ±14.38	193.6bcd ±19.72
	'Soroksár'	340.2bc ±27.49	308.1d ±27.34	167.9d ±26.51	294.4a ±40.57
Poznań	'Gold Leaf'	354.7bc ±21.22	244.1f ±18.88	270.4bc ±44.4	222.4b ±29.42
	'Lemona'	410.8a ±56.57	289.6de ±36.39	331.9ab ±22.27	205.5bc ±21.36
	'Lorelei'	-	316.3cd ±38.12	221.3cd ±23.67	163.5cd ±19.86
	‚Quedlinburger Niederliegende'	369.0b ±32.54	321.5bcd ±40.14	410.2a ±27.07	220.2b ±12.71
	‚Soroksár'	364.4b ±27.14	300.8d ±37.91	249.5bcd ±51.24	146.9d ±19.18
$LSD_{0.05}$		36.8	41.2	98.9	54.0

Table 5 .Total flavonoid content (TFC mg QE/g dw.±sd.) of lemon balm cultivars in four phenological phases in two locations (2015)

Location	Cultivar	Phenophase			
		vegetative	budding	flowering	**after flowering**
		TFC mg QE/g dw.±sd.			
Soroksár	'Gold Leaf'	0.239c±0.011	0.222d±0.027	-	-
	'Lemona'	0.648ab±0.0022	0.547c±0.0068	0.463b±0.0104	0.523ab±0.002
	'Lorelei'	0.693ab±0.087	0.560c±0.0068	0.414b±0.016	0.299de±0.014
	‚Quedlinburger Niederliegende'	0.624ab±0.0038	0.498c±0.0101	0.389b±0.0379	0.223e±0.022
	'Soroksár'	0.641ab±0.029	0.482c±0.0026	0.407b±0.0203	0.303d±0.0093
Poznań	'Gold Leaf'	0.520b±0.015	0.393cd±0.022	0.354b±0.025	0.409c±0.026
	'Lemona'	0.736ab±0.0093	0.852b±0.036	0.617a±0.0326	0.432c±0.0034
	'Lorelei'	-	0.871b±0.031	0.723a±0.0042	0.543ab±0.013
	‚Quedlinburger Niederliegende'	0.752a±0.0004	0.854b±0.043	0.663a±0.0104	0.573a±0.0021
	‚Soroksár'	0.667ab±0.049	1.152a±0.0255	0.644a±0.0261	0.473bc±0.015
$LSD_{0.05}$		0.23	0.27	0.123	0.073

RA content of the samples showed **decreasing** tendency in both locations (**Figure 4**.). The tendency through the vegetation period and the accumulation level of RA in the different phases were similar for the two locations.

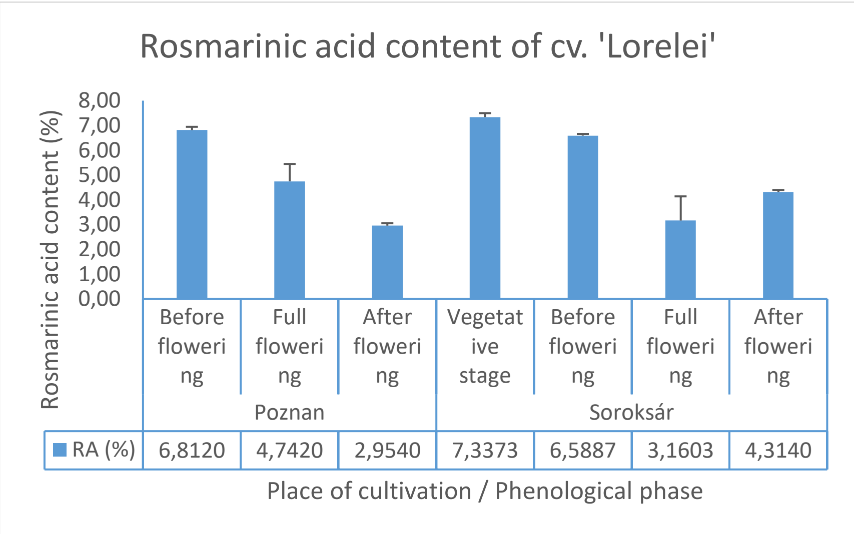


Figure 4. Rosmarinic acid content in lemon balm in different phenological phases at two locations

In thyme, TPC has been found to increase by the appearance of the generative organs (**Figures 5 and 6**.). Although the individual values of the samples vary on a wider scale, the tendency seems to be obvious: TPC is growing –with fluctuations- as the vegetation time is going on. This is a clear difference to lemon balm. At the same time, similarly to the other species, significant interaction between the variety and the accumulation tendency of TPC was found in thyme, too. The tendency has been the same at both growing locations.

Figure 5. Total phenolic content (mgGSE/g) of thyme varieties during developmental phases (Budapest, 2015)

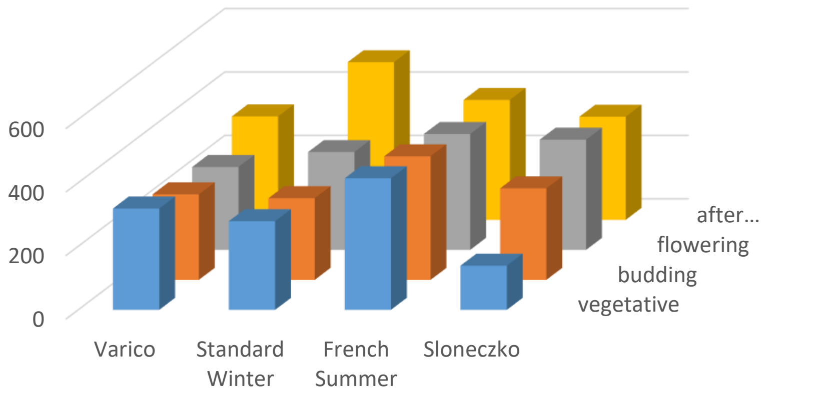
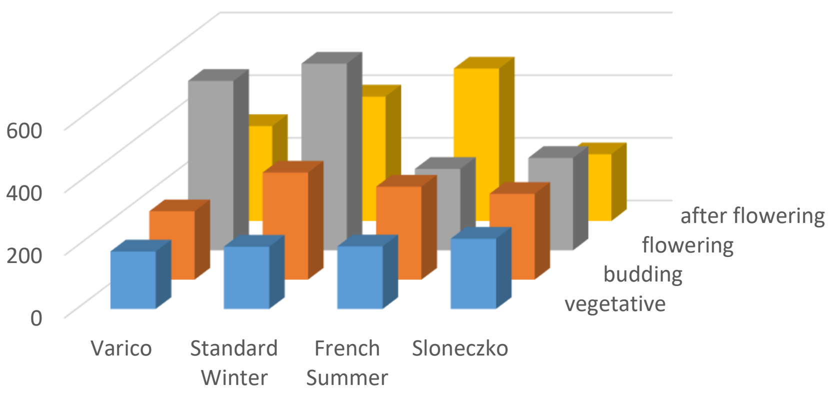


Figure 6. Total phenolic content (mgGSE/g) of thyme varieties during developmental phases (Budapest, 2016)



The described results are in harmony with the findings of the first experimental series conducted at our research station (2014, presented above). That time we also found, that the TPC of thyme increased during the sampling times. As this increase was detected both in the stressed and control plants, it could be supposed that there are ontogenetic changes in the background (**Figure** 7.).

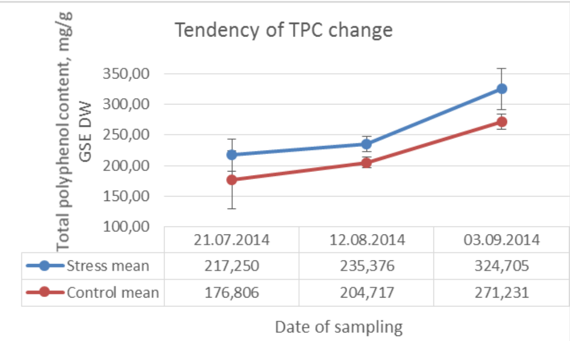


Figure 7. Content of total phenolics during the experimental period in stressed and control thyme plants (mg/g GSE)

In case of the TFC, we detected a decreasing tendency during the phenological phases. Depending on year the decrease may reach 10-43% from vegetative stage till the phase after blossoming, practically independent from the intraspecific variety. This behaviour is similar to the one established for lemon balm. The same tendency has been detected at both growing habitats.
In parallel with the accumulation of the flavonoids, the most important phenolic type compound, RA showed a definite decrease in its content during the developmental phases of thyme (**Figure 8**.). Cutting the plants at the end of flowering period only 42-75% of the initial level of RA may be present in the drug. The effect of growing location on this tendency has not been significant, similarly to the above mentioned parameters.
Taking into consideration the opposite tendencies of the levels of the studied phenolics in thyme, a shift in the spectrum of components can be supposed: possible degradation of flavonoids and RA in parallel with synthesis of other phenolic compounds. Further detailed research seems to be needed to clear up the connection among the revealed tendencies in the accumulation of phenoloid compounds of lemon balm and thyme.

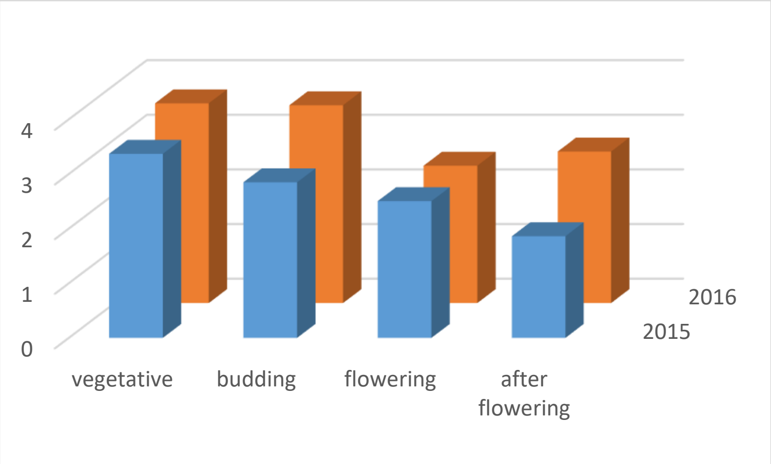


Figure 8. Rosmarinic acid content (% DM) in thyme shoots during the vegetation time (Budapest)

4. ***Changes in morphology and production as results of water shortage***

In each of our experiments (except the first one in phytotron chambers) significant changes in the studied **morphological features** have been registered due to a limited water supply.
Plant height, plant width, length and number of shoots as well as internode length were the highest in the case of the control (70% SWC) water supply while constant low soil water content (40%SWC) resulted in the lowest values. The characteristics of the plants suffering by the lower water supply reached frequently not even the half of those of the plants with control/better water supply. As a summary of the data, the following proportions can be mentioned. In plant height the stressed plants exhibited 44-77% of the values of the control ones. In case of plant with (diameter) it was 46-68%, for the number of shoots we registered 39-44% and for the length of internodia the value was 52-72%. Interestingly, these proportions did not differ considerably between lemon balm and thyme, thus, the two species showed similar levels of morphological changes due to the treatments.
To the contrary of the above listed morphological characteristics, the size of the leaves and the leaf surface (this latter one determined only for lemon balm) did not show consequent reactions in parallel with water supply. We suppose that by reduced growth it was the number of leaves which was more strongly affected (however, this trait has not been controlled in our

experiments) resulting in reduced photosynthetic and transpiration surface.
Evaluating the treatment when the low water content in the soil was applied only for a shorter period (3 weeks) we detected different results in the two pot experiments in the two years (2015-16). In 2015 for each traits this treatment showed significantly lower values than the control. Under the conditions of the second year, the findings did not differ significantly from the control in the majority of characteristics. Nevertheless, in harmony with our assumptions, the values of this treatment were most frequently between the values of the control and the continuous stress treatment. It may be assumed that the consequences of this shorter limitation of water supply are in connection with other factors and the results might depend on the weather condition and plant developmental phase, too (see also later).
In the last year we tested also the regeneration behaviour of our target plants, as mentioned above. Concerning growth and morphology of lemon balm, we experienced an accelerated growth of the plants which had been grown in severe drought conditions formerly and their measured parameters increased during the three weeks of regeneration. Although this period obviously was not enough to reach the full size of the control plants which also exhibited further growth, the tendency shows that a regeneration from the point of view of plant height and plant width is possible. Moreover, in case of the number of branches no significant difference was present among the treatments after the three weeks. In thyme, the tendencies were somewhat different. The six week drought period affected obviously so seriously the plants that no detectable change in their parameters could be measured after the three weeks regeneration period, either. In this context we have to add, that this year the size of the thyme plants was smaller than previously, thus the age of the target plants might have influenced their tolerance behaviour, too.
As an interaction of variety and stress, important observations have been made, too. Based on the results of these trials we could establish that the differences among intraspecific varieties concerning their morphological data were manifested only under optimum water regimes, while under drought the values reflected almost no significant variation and the different varieties seemed to merge into one homogeneous basic population representing the species.

Lower soil water content significantly decreased **the production** of our experimental species in each experiment (Németh et al., 2016; Radácsi et al., 2016; Szabó K. et al, 2017 submitted; Németh et al., 2017 submitted). In the first pot experiment with several genotypes in 2014 we found a larger difference in harvested biomass due to the drought treatment in lemon balm (4.47-fold) but the loss is also considerable in case of thyme (3.30-fold). These results showed that – as anticipated –, plants of larger leaves and transpiration surface are suffering more from the shortage of water. The significant loss in biomass of thyme is to some extent a surprise as it is a species of typical xerophyte properties originating of dry habitats. Our data, however, show that in low soil water content both growth and leaf development of these species is severely retarded.
It is important to emphasize, that although the biomass decreased for all cultivars under drought stress, but the degree of loss was genotype (variety) specific in both species. Similar data had been lacking till now concerning our target species.
In the climatic chamber experiment 2015 we could reveal the consequences of another situation: that of the continuously decreasing water levels to the behaviour of the plants. We established that even if the water content of the soil is decreasing stepwise and not suddenly, the loss of biomass in lemon balm is considerable. 85% of the shoot fresh mass of that of the control was measured in this treatment, while the proportion in the continuous stress treatment was 40%. These proportions in thyme are even smaller: 59% and 34% in the mentioned two treatments, respectively. This phenomenon is in contradiction to the fact that changes of phenoloid type active ingredients in this treatment could not be registered in either of the species (discussed

above). Thus, morphology and yield is a potentially better marker of the limited water supply for this plants than are the investigated phenolic constituents. These reductions might be in connection also with the unchanged (between 0.127- 0.135 g DW·g water^{-1}·100) water use efficacy as it has been calculated in a phytotron experiment for lemon balm (Radácsi et al., 2016).
Investigations on the effects of different duration of the limited water supply (2015, 2016) further on showed, that a shorter (3 weeks) water deficiency may represent another situation than long term (6-12 week water deficiency) also from the aspect of yield. In contrary to the treatment when the drought lasted for six weeks, in the case of the shorter stress period the reduction of the measured parameters were not uniform in the two years. It may show that after a stable water supply the decreasing soil water content does not necessarily affect the plants as a sudden shock but physiological changes may carry on for a longer time. The length of this transition period may depend on several circumstances. Comparing the weather conditions in the two years, we could establish that under the experimental circumstances even smaller differences in temperature and air humidity might have influenced the stress tolerance of the plants.
Data on the regeneration behaviour of these species after drought stress have been lacking till now. Based on our results, it can be established that even after a reduction of 30% in plant height and 58% of shoot mass of lemon balm the process might be reversible and an improved water supply (water content 70% SWC) accelerates growth and production reaching the values of the control plants grown under continuous optimal water supply in some weeks., however, this needs most likely a longer time for thyme.

5. *Changes of essential oil in consequence of drought stress and its relation to changes of the phenoloids*

Lemon balm and thyme are both important EO producing plants. Therefore data on the regularities of accumulation and profile of the EO have been the target of more studies formerly, especially in case of garden thyme. Nevertheless, information of the EO charateristics in consequence of changing water supply have been contradictious and about the connection of EO to changes of other ingredients (phenoloids) practically no data have been available.
In lemon balm, different varieties responded differently to the treatments. Three of the cultivars (‘Gold Leaf’, ‘Lorelei’ and ‘Quedlinburger Niederliegende’) showed the same EO content both in control (70% SWC) and stress (40% SWC) treatments. The cultivar ‘Lemona’ produced only 35% of its EO content under water stress, however cv. ‘Soroksár’ reacted with 58% increase of EO accumulation to drought treatment (Szabó K. et al., submitted 2017). Nevertheless, we have to add that the EO content of lemon balm is relatively low in general, therefore changes are difficult to evaluate.
The content of volatile compounds did not show any well defined tendency due to the stress treatment in 2015 and 2016 (Németh et al., submitted 2017). Water shortage seemed to decrease the accumulation rate in the first year but the result was an opposite one in 2016. In the oil composition of lemon balm, no significant qualitative and quantitative changes were detected between the treatments (Németh et al., 2016). Based on these findings, no connection between the level of EO and the concentration of phenoloids as well as between their changes in consequence of water supply could be ascertained in lemon balm.
In thyme, the accumulation of volatile compound has been significantly decreased in consequence of the deficiency of water (Pluhár et al., 2015; Szabó D. et al., submitted 2017). While the amounts of phenolic constituents were generally increased as a result of water deficiency, its negative effect was observed when measuring the concentration of the EO in different thyme accessions involved, irrespective of their chemotypes (**Figure 9**.). In general,

essential oil levels decreased by approx. 20 % in stressed plants of TV17, TV115 and Varico 3, except for TV143 where there was no considerable change at the last sampling time, either.
In the next year, plants suffering for six weeks by lack of water produced only 4,304% DW. EO while the ones growing in the control pots accumulated almost 6% in their leaves (2015). The plants which received the lower water supply only three weeks long exhibited medium accumulation levels, their value was 5,253% as a mean. This tendency could be ascertained also in the last year with the similar treatments and let us conclude that a longer drought situation may result in more drastic loss of EO compared to a shorter period.

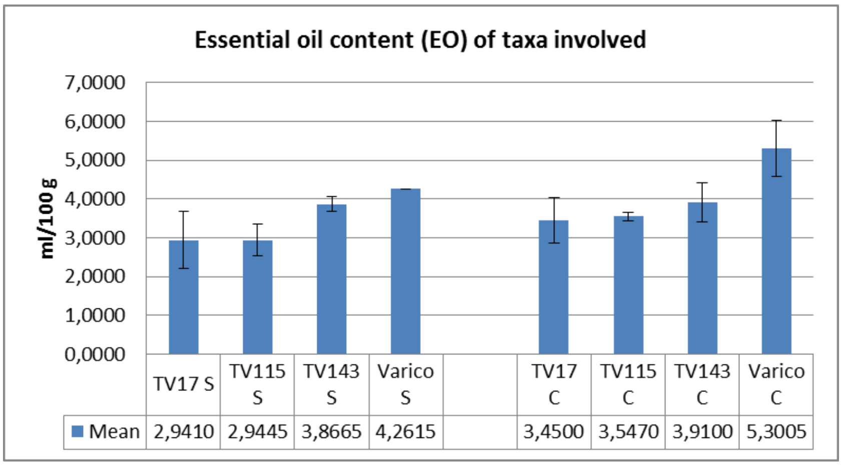


Figure 9. Effect of drought stress (C: control of 70% SWC; S: stressed of 40 % SWC) on different thyme chemotypes concerning essential oil content (EO) (ml/100 g DW) (Budapest, 2014)

As for the oil composition, no qualitative (spectrum) differences were detected due to the drought treatment, similarly to lemon balm. In thyme oil however, drought stress significantly decreased the concentrations of the main component thymol by 15.5%. Carvacrol, the related alcohol, showed a slight decrease due to the drought, while p-cymene and γ-terpinene as immediate precursors of the formerly mentioned compounds increased significantly, by 7.2% and 9.6%, respectively. It seems, that in thyme EO drought stress hampered the formation of thymol from its precursors. In other accessions however, the reaction manifested itself differently. In the EO of TV115 strain (geraniol chemotype) the ratio of geraniol was lower (40%) in the stressed plants compared to the control ones (61%) and in parallel, that of the linalool increased. Therefore it was established that essential oil composition of thyme, as a manifestation of chemosyndrome, is highly affected by water supply, but different tendencies can be present according to the original chemovariety and the respective biosynthetic relations of chief compounds.
In general, we established that the most important biologically active secondary compounds of thyme are each affected by the water supply of the plants. While the concentration of phenoloid type ingredients –including RA- is mostly increasing under drought conditions, the EO accumulation is decreasing and also unfavourable compositional changes may happen.

6. *Genetic background of rosmarinic acid accumulation-expression of the RAS gene as in drought stress situation*

According to the work plan, the investigations on the expression of RAS (rosmarinic acid synthase) gene with parallel detection of RA levels in the samples have been carried out both in phytotron as well as in pot experiments (conditions described earlier) thorough the experimental years. After optimizing the methods with standard plant material we started with the first series of experimental samples. At this step in case of *Melissa officinalis* we were faced with an unexpected difficulty: no RNA could be extracted from the leaf samples of stressed individuals. After isolation of RNA immediately during addition of LiCl, an unknown, pink-white coloured precipitated material has been developed which we were unable to eliminate and thus, no further work was possible with these samples. Several times we repeated the procedure from different plant individuals finding that in some cases the mentioned problem does not exist, however, especially in case of the stressed plants, extraction of RNA proved to be unsuccessful. We also tried to use different kinds RNA extraction kits and CTAB based protocols without success. Therefore, in the followings we focused our efforts on thyme.

Samples were taken individually and in each experiment 3-4 times during the study. RNS was extracted by CTAB method and after treatment with DNase and equalisation of the concentrations, cDNS has been synthetised. The expression of RAS was measured by rtPCR, primers were designed based on *Melissa officinalis* RAS gene sequences available from genebank. As reference gene *EF1α* was used. The amplified regions were cloned, sequenced, compared to the *M.officinalis* sequences and analysed by BLAST. Amplified region is 152 bp long, contains 14 single nucleotid polymorphism while at amino acid level it differs from *M. officinalis* sequence only in two positions.

In the climatic chamber experiments we applied four different water supplies. RAS expression of the plants suffering under the lowest soil water content (SWC 40%) was high during the full period. The group of plants which was grown at 70% SWC at first then the level decreased suddenly to 40% SWC reflected this in the RAS expression which has been relatively low at the beginning and elevated when the water level decreased. The RAS expression of the plants which got a gradually decreasing water supply increased gradually during the sampling times. This picture is however mixed considering the results of the control group where we have detected a gradually increasing RAS expression rate, too.

In the next year the same treatments had been repeated in two phytotron chambers where different temperature regimes were installed in order to check the interaction of water supply and temperature- according to our research plan. These regimes were 25/18°C and 20/15°C day/night temperatures.

Under the „warm" circumstances control plants showed a slightly decreasing and afterwards a slightly increasing values both for RAS expression and RA concentration (**Figure 10**.). To the contrary, RAS gene expression of the continuously stressed plants showed at the beginning an incline, after that a decline and at the end the activity increased again. After an increase at the beginning, RA content decreased in these samples which is only in partial harmony with the mentioned gene activity, however a time shift between gene expression and RA accumulation should be considered. Plants getting the gradually decreasing water supply reflected almost the same behaviour but at the end their values decreased again. Plants which got only at the end a lower water supply exhibited a decreasing RAS expression with an elevation however after the dryer soil was present. The level of RA in these last two treatments showed a parallel tendency with the expression of its gene. In the phytotron with the lower temperature regime we found to some extent different patterns first of all in case of the control plants, where a big decline of RA content was measured at the last sampling without a parallel tendency in its gene expression.

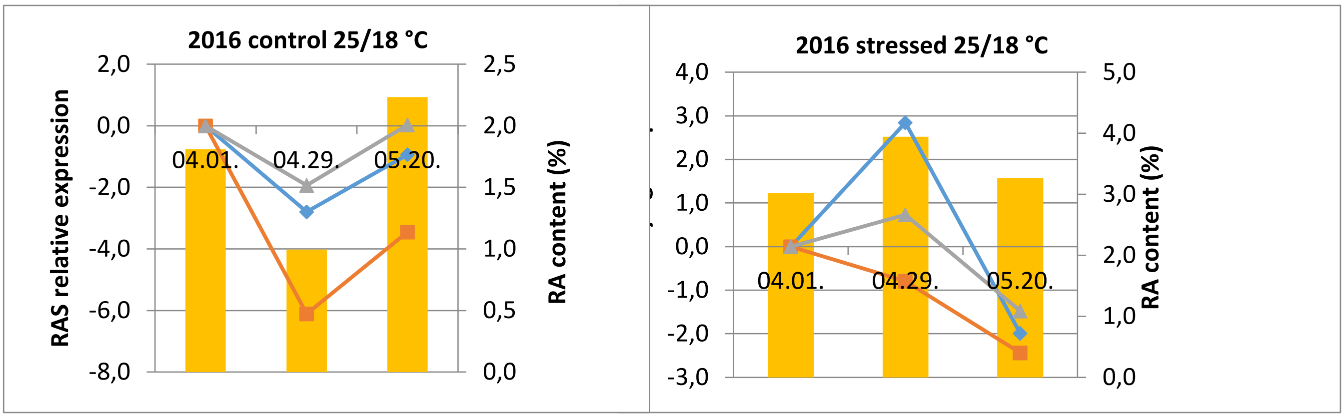


Figure 10. RAS gene expression and RA content of thyme in phytotron experiment, "warm" chamber. *Columns: RA content; Lines: three plant individuals*

Altogether it was detected in these experiments, that plants grown at a lower saturation of SWC accumulated by 1.5-4 more RA than did the control ones. It might have a practical significance in the cultivation. The expression of RAS gene seems to be in harmony with this accumulation tendency. Nevertheless, there are unexpected fluctuations which might be the result of different factors. Based on the data, temperature is an important factor influencing the accumulation dynamics and level of RA (being lower at colder temperatures). Besides, individual variations among the plants both in RA content and RAS gene expression were obvious. Thus, bulk samples of the experimental population may cover the individual tendencies. At the same time the amount of the experimental plant material did not allow us to use individual samples in each case which seems to be a problem to take into consideration during further similar works.

In the trial under semi-controlled conditions different results were achieved in the two years. The first one showed practically no difference in RAS gene expression level in either of the individuals independent from drought treatments. In 2016 we found, that in the control plants RAS expression decreased, afterwards increased and at the end decreased again very sharply. On the other side, stressed plants (40% SWC) demonstrated an elevation first, a decrease afterwards which was followed another increase of the activity. The plants which got only a shorter drought period in the second half of the time showed also an increase to the end of the trial. The mentioned behaviour practically in agreement with the findings in the former phytotron experiment. It can be concluded that the expression of RAS gene and –presumably- the production of corresponding enzymes is not a permanent process but undergoes certain fluctuations during plant development which may be explained by acclimatization process, plant development and alternative biosynthetic mechanisms. Differences among plant individuals were also observable. Altogether it was established that in case of lower water content of the soil, the expression of RAS gene is elevating.

A further aspect of our studies was following the tendencies of RA accumulation during the developmental phases. Therefore, RNA was extracted from thyme in different phenological phases, too. In each phase (vegetative, budding, full flowering, seed ripening) three selected plant individuals were sampled. In both study years all of the individuals exhibited a similar expression pattern. In the year 2015 after the high level at the beginning, the expression decreased during flowering until a hardly detectable level and it increased only at the end of the sampling period again. In the second year, highest expression has been detected in budding stage after which it dropped and at the end of flowering showed an elevated activity again. This is only partially in harmony with the RA content measured in the same plants. As described in Chapter 3 above, RA content of thyme decreased permanently during the phenological phases in both years. Thus, it seems to be likely that beside transcriptional regulation, other genetic or enzymatic processes are influencing the level of RA in this plant. Furthermore, we also mentioned, that in parallel to the changes of RA, TPC showed an increasing tendency towards the end of the vegetation period. In this context, it would be interesting to study the detailed phenolic spectrum of thyme during this phases as the concentration of RA might be determined not only from the side of its synthesis (gene expression) but also the concurrent metabolic processes.

7. General conclusions and practical aspects

Differences between the two target species manifested themselves in the level of the studied polyphenolic compounds, in general, lemon balm accumulating higher values. Concentration of phenolic compounds reflects intraspecific differences among accessions of thyme but lemon balm shows a much lower intraspecific chemical variability concerning these compounds.

It was detected that the reaction on water shortage might be totally different even in related MAP species. Phenols are not obviously present as stress defence compounds in each taxon. In lemon balm longer term drought and decreasing water supply did not induced significant changes in the accumulation of phenoloids and could be concluded that the level of total phenoloids and that of RA most likely do not participate in defence against low soil water content. In thyme, TPC and TFC increased as the drought became sever supporting the idea that polyphenols may play a role in drought stress response. RA exhibited the highest value in short term stress treatment, thus its increase might be a sudden reaction of thyme to water deficit. Thus, the higher tolerance of thyme might be the result of more flexible defence mechanisms and in accumulation of phenolic type special compounds.

In thyme although the rate of the change between control and stressed plants reflected genotype-dependence, the direction of these changes was the same in each accession. This is also a clear difference compared to lemon balm, where in most cases even the direction of the changes was not uniform.

In lemon balm no connection between the level of EO and the concentration of phenoloids as well as between their changes in consequence of water supply could be ascertained. In thyme, the accumulation of volatile compound has been significantly decreased in consequence of the deficiency of water and the longer drought resulted in more severe loss. Besides, essential oil composition is also affected by water supply, but different tendencies can be present according to the chemovariety.

Interestingly, lemon balm reacted to an improved water supply after the stress period much quickly, three weeks regeneration period assured significant improvements in its biomass. Thyme however presumably would need a longer regeneration period for re-growth.

Similarities in the reaction of the two target species were also observed. During their phenological phases, the content of phenoloids changed. This change was almost in each case a decrease, except TPC in thyme which increased as the plant reached the generative phases.

The growing location affected in both species the level (concentration) of the measured parameters and had practically no influence on the accumulation dynamics which therefore, might be a more genotype-dependent feature.

Significant interaction between the varieties and the accumulation tendency of total phenolic compounds was found as a further common characteristics of both species.

In each of our experiments significant reduction of the studied morphological features and biomass have been registered due to a limited water supply. In this respect the two species showed similar magnitude of changes due to the treatments.

For the **cultivation practice** it should be important, that even thyme, this xerophyte plant may suffer by low water supply and its drug production would be significantly reduced. Beside this,

the concentration of phenolics –among them also that of RA- may increase and in parallel the content of the volatiles decrease with additional compositional changes which result in a significantly changed drug quality.
Lemon balm seems to react to the drought primarily with reduced growth and yield –at the same time with extended root mass- however, active ingredients of the drug are less threatened.
An important aspect of our results is that different varieties of lemon balm and thyme may exhibit to some extent different tolerance to the reduced water supply and the specific chemical characters of them could be manifested only under optimal water supply.
Our data suggested that temperature is a basic influencing factor even on a short run in the stress reaction of these species, including morphological changes but also RAS gene expression. The same soil water level might induce defence against drought stress however, at lower temperatures might represent more optimal water regime. This aspect needs further studies in controlled environments.
In the cultivation practice of MAPs, field of lemon balm and thyme are hardly irrigated Therefore it can be important to see the consequences of the drought stress period in open field.

Publications prepeared from the results till nowhave been uploaded to the website. In 2016 we have applied to NKFIH for an extended finel publication deadline of 31 December 2017. Till this time we hope, the following additional manuscripts would appear.

Further manuscripts submitted to journals:
1./ Éva Zámboriné Németh, Péter Radácsi, Beáta Gosztola, Péter Rajhárt, Krisztina Szabó: Influence of water supply and its fluctuations on yield and quality of lemon balm (*Melissa officinalis* L.). Australian Journal of Crop Science (March, 2017)
2./ Krisztina Szabó, Péter Radácsi, Márta Ladányi, Éva Németh: Stress-induced changes of growth, yield and bioactive compounds in lemon balm (*Melissa officinalis* L.) cultivars. Environmental and experimental botany (April, 2017)
3./ Dóra Szabó, Michael Abiodun Falade, Péter Radácsi, Katalin Inotai, Zsuzsanna Pluhár: Effect of water deficit on growth, yields, active compounds and antioxidant capacity of (Thymus vulgaris L.), Industrial Crops and Products (April, 2017)

Manuscripts in preparation
1./ Katarzyna Seidler-Łożykowska, Krisztina Szabó, Péter Radácsi, Jan Bocianowski, Eva Nemeth-Zamboriné: Effect of location on morphological, chemical traits and antioxidant activity of lemon balm (*Melissa officinalis* L.) cultivars
2./ Zsuzsanna Pluhár, Dóra Szabó, Chemical composition and antioxidant activity of different *Thymus vulgaris* chemotypes influenced by drought stress
3./ Dóra Szabó, Péter Radácsi, Zsuzsanna Pluhár: Az eltérő hőmérséklet és vízellátottság hatása a kerti kakukkfű (*Thymus vulgaris* L.) produkciójára és morfológiai tulajdonságaira
4./ Dóra Szabó, Katalin Inotai, Katarzyna Seidler-Lozykowska, Éva Németh-Zámboriné, Zsuzsanna Pluhár: Changes in essential oil properties of different (*Thymus vulgaris)* varieties influenced by developmental phases, growing locations and seasons