**Final Report- OTKA PD 105750 (2013-2016)**
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# Potential effect of arbuscular mycorrhizal fungi on the growth and the secondary metabolite production of marigold, marjoram and lemon balm

## BACKGROUND

***Arbuscular mycorrhizal fungi*** (AMF) occur in all terrestrial ecosystems and form symbiotic associations with the majority of terrestrial plant species, including many agriculturally and horticulturally important crop species.[1] Botanically the arbuscular mycorrhiza (AM) is a mutualistic relationship between phytobionts and mycobionts. AMF have direct and indirect effects on plant nutrient uptake, root morphology, many physiological and developmental processes of plants.[2] These fungi are obligate biotrophs, which must develop a symbiotic association with the host in order to grow and complete their life cycle.[3] AM fungi take up products of host plant photosynthesis. Hexoses transfer may occur through arbuscules as an intracellular interface of symbiotic association. In contrast to the diverse array of terrestrial plants that form AM, only ca. 200-250 *Glomeromycota* species have so far been described, suggesting that mycobiont specificity is low relative to their potential plant hosts.[4] The root colonization by AMF has been found to increase the productivity of several crops due to a range of benefits provided to the host plants. Positive effects of AM symbiosis on plant growth, water uptake, nutrition uptake and tolerance to environmental stressors (drought, salt, heavy metal pollution, soil pathogens) have been proved in numerous studies.[5-7] Apart from these beneficial effects, accumulation of secondary metabolites such as essential oils, alkaloids and phenolic compounds in medicinal plants can also be affected by this symbiosis inducing important changes in physiological processes of host. [5,8,9]
***Polyphenols*** form a diverse class of plant secondary metabolites (e.g. phenolic acids, flavonoids) widespread in plant kingdom. When they are consumed as part of a diet in various forms of polyphenol-rich plant foods, several biological activities are attributed to them.[10] For instance, polyphenol rich diet can play an important role in the prevention of various diseases such as cardiovascular and neurodegenerative disorders.[11,12] Besides fruits and vegetables, many herbs and spices serve as excellent sources of phenolic compounds[13], among them ***pot marigold*** (syn. poor man saffron, *Calendula officinalis* L., *Asteraceae*), ***sweet marjoram*** (*Origanum majorana* L. = *Majorana hortensis* Moench., *Lamiaceae*) and ***lemon balm*** (*Melissa officinalis* L., *Lamiaceae*). These three herbs are native to the Mediterranean region and worldwide cultivated for culinary, medicinal, cosmetic and ornamental purposes.[14-16] Pot marigold, sweet marjoram and lemon balm used in the food and pharmaceutical industry are mainly gained from cultivation where several growing conditions can be controlled. With the development of new agricultural techniques, the yield and quality of plant material can be improved significantly to fulfill the increasing industrial and consumer demands. One of the promising possibilities for enhancing the biomass and increase the quality of herbs can be the application of arbuscular mycorrhizal fungi during their cultivation.
The ***main aim*** of this investigation was to examine the potential effects of arbuscular mycorrhizal fungal (AMF) colonization on growth and polyphenol content of three traditionally and economically important herbs, such as pot marigold, sweet marjoram and lemon balm.

## MAIN MATERIALS AND METHODS

**Plant material:** ***Pot marigold***, ***sweet marjoram*** and ***lemon balm*** were examined in this study since these species favourably form symbiotic relations with AMF, and they are commercially important herbs. In addition, they are considered as excellent and diverse model species since they represent two different plant life-forms (annual and perennial) and moreover, different parts of them are utilized. Namely, flower of marigold, flowering shoots of marjoram and leaves of lemon balm are used as plant drugs, i.e. the parts rich in active substances. In order to ensure the maximal homogeneity of the plant material, three reliable and commercially available cultivars were chosen for this study: *Calendula officinalis* 'Calypso Orange with Black Eye', *Melissa officinalis* 'Relax', *Origanum majorana* 'Esperanta'.

**Planting:** In the case of open and semi-open field experiments seeds were planted into plotting soil sterilized by autoclave. To support the mycorrhization of the plants, special, reduced phosphorus content soil was applied (NPK 12:11:17). The sterile and inoculated plants were grown in propagation trays. The seedlings were planted to the experimental field of the Centre for Ecological Research, Hungarian Academy of Sciences at Vácrátót at the beginning of the summer.

**Inoculation:** The inoculation with AMF inoculum was done during the planting. Two commercially available inocula were used for the directed mycorrhization of the herbs: ***INOQ –top*** (mixture of *Claroideoglomus etunicatum*, *Rhizophagus intraradices*, *Claroideoglomus claroideum*) and **Symbivit** (mixture of *C. etunicatum*, *G. microaggregatum R. intraradices*, *C. claroideum*, *Funneliformis mosseae*, and *F. geosporum*).

**Harvesting:** ***Flowers*** of marigold collected weekly, ***flowering shoots*** of marjoram collected twice, ***leafy shoots*** of lemon balm collected at the end of the vegetation period. Fresh and dry weights of collected plant material were determined. After drying procedure the sepals were removed from the flowers of marigold, the stems were removed from the collected shoots of marjoram and lemon balm. These purified plant material were used for chemical analysis.

**Root colonization:** The efficiency of the directed mycorrhization was verified by monitoring the root colonization of the plants. For this purpose root samples were taken three times during the vegetation period. Following the staining procedure of the root samples, the characterization and the evaluation of the AMF root colonization parameters were performed using the method of Trouvelot et al. (1986)[17]. Based on the examination of the chosen root pieces the frequency and intensity of the mycorrhizal infection (F %; M%) and the arbusculum content of the infected parts (a %, A%) were calculated. AMF propagation, assessment of fungal colonization and soil analyses were carried out at the Institute for Soil Science and Agricultural Chemistry, Centre for Agricultural Research, Hungarian Academy of Sciences.

**Determination of phenolic compounds:** Identification of the major polyphenolic compounds was performed with a high performance liquid chromatography (***HPLC***) system including a diode array detector (DAD) coupled to an quadrupole – time-of-flight mass spectrometer (q-TOFMS), which was equipped with a dual spray ESI source. Quantitative determination of the identified compounds was accomplished with an optimized gradient grade chromatographic method using HPLC-DAD system[18].

## MAIN RESULTS

### Identification of phenolic compounds

The following phenolic compounds were tentatively identified based on UV spectra and accurate mass fragmentation information in the case of the three herbs:

Marjoram: Apigenin-6,8-di-*C*-glucoside, Luteolin-7'-*O*-glucuronide, Rosmarinic acid, Apigenin-glucuronide, Lithospermic acid A isomer (a), Salvianolic acid B, Apigenin

Lemon balm: Caftaric acid, Caffeic acid, Caftaric acid hexoside, Rosmarinic acid, two Lithospermic acid A isomers (a, b), Luteolin-3'-*O*-glucuronide

Marigold: Chlorogenic acid, Quercetin-3-*O*-rhamnosyl-rutinoside, Isorhamnetin-3-*O*-rhamnosyl-rutinoside, Rutin, Isorhamnetin-3-*O*-neohespedidoside (calendoflavoside), Isorhamnetin-3-*O*-rutinoside (Narcissin), Dicaffeoylquinic acid, Isorhamnetin-malonyl-glucoside

The main compounds of marjoram and lemon balm were rosmarinic acid and lithospermic acid A isomers, while in marigold isorhamnetin-3-*O*-rhamnosyl-rutinoside and narcissin were the most abundant ones. Interestingly luteolin-3'-*O*-glucuronide was missing from the lemon balm plants grown in plant-growth room[19].

### Results of colonization, biomass and polyphenol content breakdown by years

## FIRST YEAR

In 2013 pot and open field experiments were performed in parallel involving marigold, marjoram and lemon balm.

### Aims and design

The aim of the **pot experiment** was to test the efficiency of the chosen, commercially available AMF inoculum excluding other environmental factors. Thus two groups of each plant species, a (i) **mycorrhizal** and a (ii) **non-mycorrhizal** control group, were grown in plant-growth room under controlled conditions.
Open field experiment was set up in order to examine the effect of AMF inoculation in the agricultural practice in comparison with the use of artificial fertilizer (NKP). For this purpose the following four treatments (including the control) in three replications were arranged in a complete random block design:

***Control***: soil of the experimental field (exp. field)
***Treatment-1***: soil of the exp. field + commercially available fungal inoculum (AMF/INOQ)
***Treatment-2***: soil of the exp. field + artificial fertilizer (NPK)
***Treatment-3***: soil of the exp. field + commercially available fungal inoculum (AMF/INOQ) + artificial fertilizer

**Main results of the pot experiment**

***Colonization***

All three herb species formed symbiotic association with the added AMF inoculum. The root system of the three plant species were colonized in different rates. According to the root colonization parameters marjoram plants had the highest level of fungal colonization (M=82%) followed by lemon balm (M=62.5%) and marigold (M=17%).

***Biomass***

Mycorrhization significantly increased the yield of flowering shoots in marjoram. The AM plants had more than 1.5 times higher biomass production compared to non-mycorrhizal plants. The AMF treatment did not cause significant differences in the yield of lemon balm (leafy shoots) and marigold (flowers). However, a significant difference was observed in the number of marigold's flowers. AMF inoculation significantly increased the number of flowers. Thus AM plants had a higher number, nonetheless smaller flowers compared to the control plants.

***Polyphenol content***

Marjoram: A significant decrease of the two main compounds, namely rosmarinic acid and lithospermic acid isomer was observed in plants colonized by AMF. These compounds were presented in 28 and 24 % lower concentration than in the non-mycorrhizal control plants, respectively than in control plants. Regardless of the lower concentration of the above mentioned compounds, mycorrhization increased the total yield of polyphenols calculating on the yield of biomass (Fig 1.).

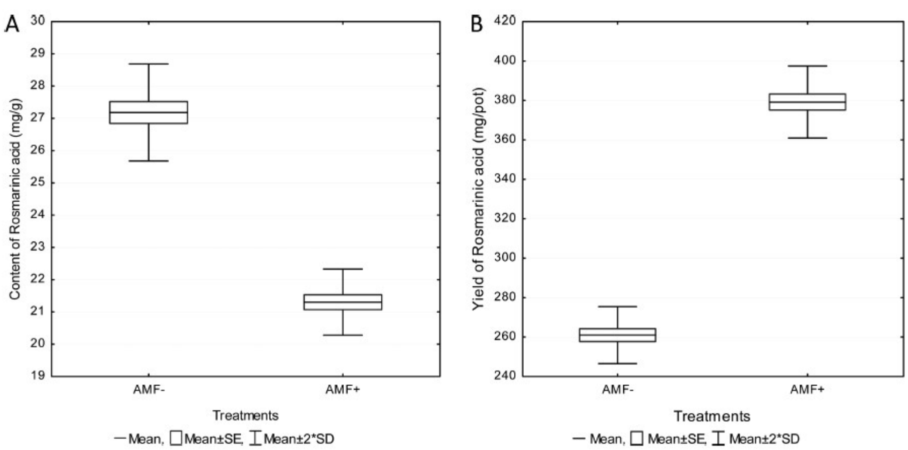


Fig.1: Box plot graphs of content (A) and yield (B, multiplying the values of content by the biomass dry weight) of rosmarinic acid in non-mycorrhizal (AMF-) and mycorrhizal (AMF+) marjoram plants

Lemon balm: The mycorrhization also influenced the concentration of phenolic acids. In contrast to marjoram, the content of the main phenolic compounds in AMF inoculated lemon balm was 11-17 % higher than in control plants. Since the mycorrhization had no significant effect on the yield of biomass, the total yield of polyphenols showed similar tendency to the content of polyphenol.

Marigold: Significant changes were only observed in the concentration of calendoflavosid and isorhamnetin-malonyl-glucoside. These compounds were present at 5-25 % higher concentrations in the flowers of AMF treated plants as compared to the non-mycorrhizal ones. However, this significant difference appeared only in isorhamnetin-malonyl-glucoside concerning the total yield.

**Conclusion**

Positive influence of AMF inoculation has been proved. AMF can provide different services for each herb. For instance while marjoram benefits more from the AMF colonization in terms of growth, lemon balm and marigold have higher polyphenol content. The level of changes caused by AMF in plant production corresponded with the intensity of mycorrhizal colonization[19].

**Main results of the open field experiment**

***Colonization***

The mycorrhizal colonization of the plants inoculated by AMF were differed significantly. Among the three herbs marjoram seems to be the most susceptible to the AM fungi- plant symbiosis. Intensity of colonization (M%) estimated in the root system of the seedlings before plantation were the following: marigold (M=6%) < lemon balm (M= 20%) < marjoram (M=43%). After plantation of the plants into the experimental field the root system of the control groups were colonized by the native AMF found naturally in the soil (M= marjoram 28%, lemon balm 16%, marigold 12%). The effect of the inoculation obviously appeared in those treatments when artificial fertilizer was not used. At the end of the vegetation period the inoculated marjoram plants had the highest level of mycorrhizal colonization (M=51%) followed by lemon balm (M=27%) and marigold (M=25%). The fertilization suppressed the mycorrhization (M= marjoram 14%, lemon balm 7%, marigold 3%). In the case of the inoculated plants the rate of the colonized roots was near the double, while in the case of the fertilized plants this rate was the half compared with the control group.

***Biomass***

Only in the case of lemon balm was significant difference among the treatments. The control plants had significantly higher biomass compared to the plants treated by the combination of fertilizer and AMF.

***Polyphenol content***

Marjoram: The aerial parts of marjoram were collected two times (beginning of August, end of September). The treatments caused significant differences in the polyphenol content of marjoram. Examining the samples from the first harvest the lowest content of active substances was observed in the case of control group. The content of the main compounds of the AMF treated plants was two times higher compared to the control group. The plants treated with artificial fertilizer and AMF (NPK+AMF) and only with NPK contained 70% and 30 % more active components than the control group respectively.

Observing the samples collected two months later the differences between the treatments were obliterated in the case of the flavonoids. On the contrary the effects of the treatments are still outstanding in the case of lithospermic acid and rosmarinic acid. Although the effects are differ from the previously observed ones. The following results were gained in reference to the content of lithospermic acid and rosmarinic acid: control group > AMF treated plants (20% less than control) > NPK+AMF treated plants (half of the control)> NPK treated plants 70-80 % less than the control). To compare the content of active substances derived from the two harvesting times a 50-70 % decrease was observed in the case of AMF, NPK and the NPK+AMF treatments during the vegetation.

The application of chemical fertilizer suppressed the production of polyphenols. The control group had the highest polyphenol content. The use of AMF/NOQ could compensate the negative effect of fertilizer. The most significant effect of the treatments was observed in the case of phenolic acids, such as rosmarinic acid. The application of chemical fertilizer suppressed the production of polyphenols. The control group had the highest polyphenol content. The use of INOQ-top inoculum could compensate the negative effect of the fertilizer (Fig.2).

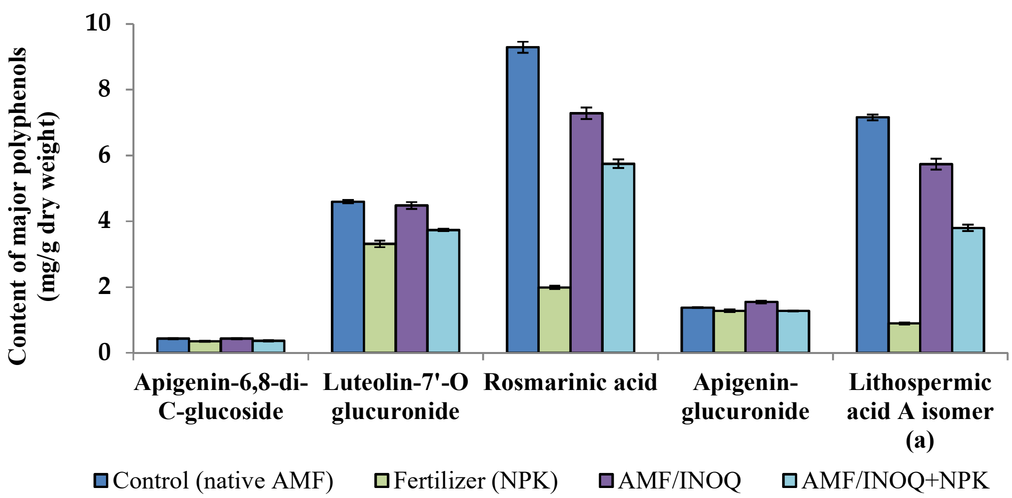


Fig. 2.: Polyphenol content of marjoram collected in September 2013. (AMF-arbuscular mycorrhizal fungi, NPK-artificial fertilizer, AMF/INOQ commercially available fungal inoculum)

Lemon balm: The treatments significantly affected the content of rosmarinic acid and lithospermic acid A isomers. Application of fertilizer stimulated the phenolic acid production. The commercial AMF also increased the phenolic acid content compared to the control group. In case of the lemon balm the NPK treatment caused the highest content (8-11 times higher than the control group) of the three main compounds (rosmarinic acid, lithospremic acid and salvianolic acid derivative). Increase in the content of the main compound was also observed due to the AMF and the NPK+AMF treatments (3-6 times higher than the control) (Fig3.).

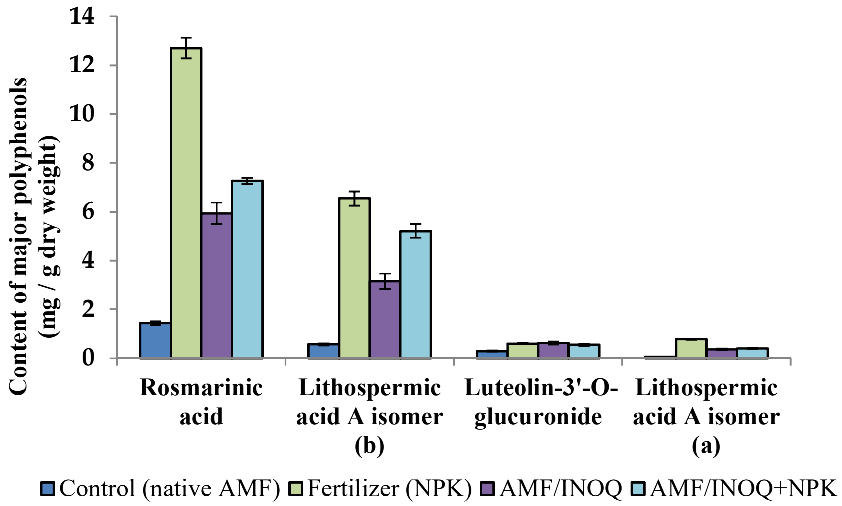


Fig.3.: Polyphenol content of lemon balm (AMF-arbuscular mycorrhizal fungi, NPK-artificial fertilizer, AMF/INOQ commercially available fungal inoculum)

Marigold: Treatments significantly modified the content of minor polyphenols. Native AMF proved to be the most effective among the treatments. The content of active substances of marigold showed similar pattern to lemon balm as the results of the treatments.

***Essential oil content***

In this year we had the possibility to examine the essential oil content of marjoram. The presence of 30 essential oil components was determined among which terpinen-4-ol, cis-sabinene hydrate, trans-sabinene hydrate, linalyl acetate, trans-sbinene hydrate acetate and p-cimol were the major ones representing the 70-78% of the total content. The results indicate that the colonization with native AMF increased the essential oil production.

In the case of the essential oil of marjoram the treatments caused a change in the rates of trans-sabinene hydrate acetate and linalyl acetate. Contradict the results of polyphenols the fertilization raised the rate of these compounds in the essential oil. While they presented in the lowest rate in the control group. Similarly to the polyphenol content the accumulation of essential oil was the highest in control group. Interestingly the simultaneous use of NPK and AMF/INOQ increased the essential oil content compares to their single use[20].

**Conclusion:**

The results are obviously reflected the positive effects of the inoculation and the negative effects of the artificial fertilizer on the formation of the symbiosis. It is also evident that the sampling time can influence the polyphenol content. The different AMF colonization rate could be one of the influential factors, which can contribute to the differences between the quality of plant materials harvested in different period. The effect of the directed inoculation on the secondary metabolite synthesis could be detected under open field conditions. It needs to be taken into account that the use of artificial fertilizer could negatively modify the polyphenol content, thus the dosage of chemicals should be optimized. In some cases the symbiotic relation formed with AMF populations found naturally in the soil could be also favourable to the production of herbs. Therefore the preparation of new inoculum from these AMF population can be prosperous to cultivation of herbs[20,21].

## SECOND YEAR

### Aims and design

In 2014 the effect of the commercially available AMF inoculum and the native AMF populations found naturally in the soil of the experimental field was compared. Besides the commercially available AMF inoculum, the three native AMF populations isolated from the rhizosphere of the three host plants were tested. Instead of one annual species (described in the work plan) both of the annual species (marigold, marjoram) and the lemon balm examined also in this year. The primer inoculums of indigenous AMF population were isolated from the rizosphere of the three host plants (control groups) planted in the previous year, and they were propagated in large scale. All the three plant species (marjoram, lemon balm and marigold) were treated with the four types of AMF inoculums. The following six treatments were accomplished in the case of the three plants with three replications:

***Control (-)***: sterile soil of the experimental field (exp. field) in pots (10L)
***Control (+):*** soil of the exp. field
***Treatment-1***: soil of the exp. field + commercially available fungal inoculum (AMF/INOQ)
***Treatment-2***: soil of the exp. field + inoculum isolated from rhizosphere of marjoram (AMF/*Origanum*)
***Treatment-3***: soil of the exp. field + inoculum isolated from rhizosphere of marigold (AMF/*Calendula*)
***Treatment-4***: soil of the ex. field + inoculum isolated from rhizosphere of lemon balm (AMF/*Melissa*)

### Main results

***Colonization***

The colonization of the native AMF inoculums was more infective than the commercially available AMF in all cases. In the term of mycorrhization the root systems of the three plants have been colonized in different level with the applied AMF inoculums. In the case of marigold there were not significant differences between the AMF treatments in the intensity of the mycorrhizal infection (M=47-58 %). While in the case of marjoram and lemon balm the AM fungal populations isolated from the rhizosphere of marjoram formed the strongest association with their root-systems (M=35 %; M=72 %, respectively).

***Biomass***

The plants planted into pots field with the sterile soil of the experimental field (negative control) had significant low biomass-production in the case of all three plant species.
In biomass-production significant increase was determined in marjoram plants inoculated with AMF/*Origanum* and AMF/*Calendula*. In case of marigold the plants inoculated with AMF/INOQ and the positive control group had the highest flower numbers. There were not significant differences between the treatments (beside the negative control) in the biomass of lemon balm.

***Polyphenol content***

Marjoram: In marjoram a decrease of the content of rosmarinic acid and lithospheric acid was detected in the plants inoculated with AMF/*Calendula*. In the other three AMF treatments there were no significant differences in the content of these components.

Lemon balm: In the chemical composition the most significant differences were observed in the phenolic acid (rosmarinic acid and lithospermic acid) content of lemon balm. The highest content of phenolic acids was in the plants inoculated with AMF/*Origanum*. The content of rosmarinic acid (41 mg/g) was two times, the content of lithospermic acid was 1.6 times higher in these plants compared to the positive control group (Fig 4.).

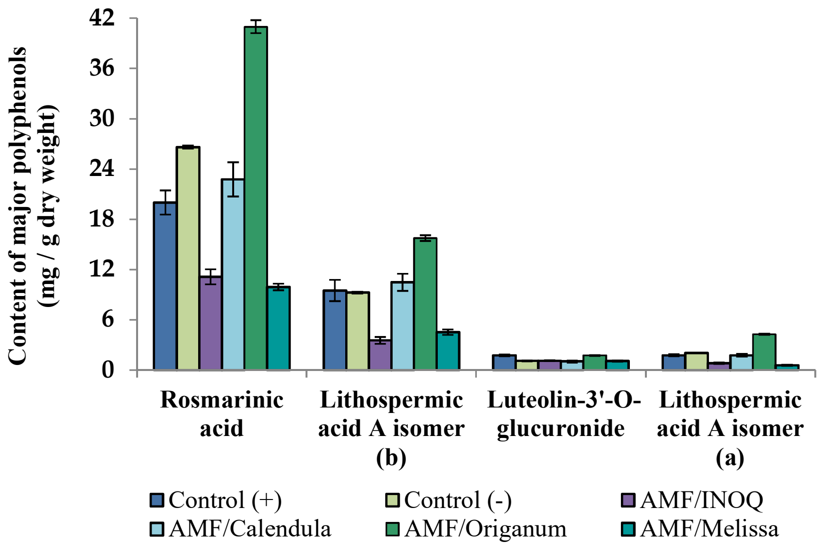


Fig.4.: Polyphenol content of lemon balm (commercially available fungal inoculum: AMF/INOQ, inoculum isolated from rhizosphere of marigold: AMF/Calendula, of marjoram: AMF/Origanum, of lemon balm: AMF/Melissa)

Marigold: The narcissin content of marigold was significant higher in the plants inoculated with AMF/INOQ compare to the plants treated with the indigenous AM fungal populations.

## Conclusion

Considering the results of the mycorrhization, chemical composition and biomass-production in the case of marjoram and lemon balm the indigenous AM fungal populations isolated from the rhizosphere of marjoram (AMF/*Origanum*), while in marigold the commercially available INOQ-top AMF inoculum was the most effective. It shows that the effect and outcome of mycorrhization depends on the plant and also the fungal species[22].

## THIRD YEAR

In 2015 the field experiment described in the work plan was extended. Instead of one annual species, both annual species (marigold, marjoram) and also lemon balm as a perennial species were planted.

### Aims and design

In 2015 doses of artificial fertilizer (NPK) were optimised to find the most effective combined application of fertilisers and AMF. Three different doses of NPK were added to the plants. The doses-1 of NPK were calculated according to the need of each plant (marigold: N 50 kg/ha, P 70 kg/ha, K 90 kg/ha P; marjoram: N 50kg/ha, P 70 kg/ha, K 130 kg/ha, lemon balm: N 60 kg/ha, P 60 kg/ha, K 70 kg/ha). The dose-2 indicates the half while dose-3 means the one-third of the dose 01.In this experiment the most effective AM fungal inocula were used (based on the results of the previous year). In the case of marjoram and lemon balm the most effective AMF inoculum was the indigenous AMF population isolated from the rhizosphere of marjoram (AMF/*Origanum*). While in marigold the INOQ-top commercially available AMF inoculum was the most convenient (AMF/INOQ).
The following seven treatments were accomplished in the case of the three plants with three replications:

***Control (-)***: sterile soil of the experimental field (exp. field) in pots (10L)
***Control (+):*** soil of the exp. field
***Treatment-1***: soil of the exp. field + the most effective AMF inoculum
***Treatment-2***: soil of the exp. field + NPK-dose-1
***Treatment-3***: soil of the exp. field + the most effective AMF inoculum + NPK-dose-1
***Treatment-4***: soil of the exp. field + the most effective AMF inoculum + NPK-dose-2
***Treatment-5***: soil of the exp. field + the most effective AMF inoculum + NPK-dose-3

### Main results

#### *Colonization*

Among the three plant species marjoram had the highest level of mycorrhizal colonization (M=30-67.9 %). While lemon balm (M=9-32.5%) and marigold (M=6.9-35.7%) had similar infection rate. The negative effect of artificial fertilizer on mycorrhization appeared in all three plant species. In the case of marigold and lemon balm all the three NPK doses suppressed the fungal colonization (marigold: M= 6.9-29.6%, lemon balm: M=9-18.6%) in comparison with the AMF inoculated plants (marigold: M= 35.7%, lemon balm: M=29.9%). In marjoram only NPK-dose-1 had negative effect on fungal inoculation (M=45.5 %). NPK-dose-2 and NPK dose-3 did not interfere the colonization (M= 67.9% and 63%, respectively), moreover in these treatment the intensity of the mycorrhizal infection was higher than in the single AMF treatment (M=55.7%). In the case of all three plant species the plants from the positive control groups formed fungal colonization with native AMF populations found naturally in the soil (marjoram: M=30.3 %, lemon balm: M=32.5%, marigold: M=27.8%).

### *Biomass*

Significant differences in biomass was detected only in a few cases. In the case of negative control groups only lemon balm had measurable biomass. The biomass production of these control plants was the third part of the plant material gained from the other treatments. AMF inoculated marjoram plants had significantly lower biomass production in comparison with the other treatments.

### *Polyphenol content*

Marjoram: The samples collected in September had higher polyphenol content than the samples collected in August. In this year the most significant differences have been detected in the content of the two main components (rosmarinic acid, lithospermic acid) of marjoram. The AMF inoculation and the addition of different doses of NPK resulted in higher rosmarinic acid content in comparison with control group. The most effective treatment was the treatment-4 when beside AMF inoculation the half of the recommended artificial fertilizer portion (NPK-dose-2) was added to the plants. These plants had near eight-time higher rosmarinic acid content than the control group. This result is in a good agreement with the results of colonization. The treatment-2 and -3 (so the addition of NPK-dose-1) resulted in four- and three-time higher rosmarinic acid content in comparison with the control plants. While the rosmarinic acid content of plants treated with NPK-dose-3 (treatment-5) was the double of the control value. Regarding the content of lithospermic acid similar tendency was observed (Fig.5.).

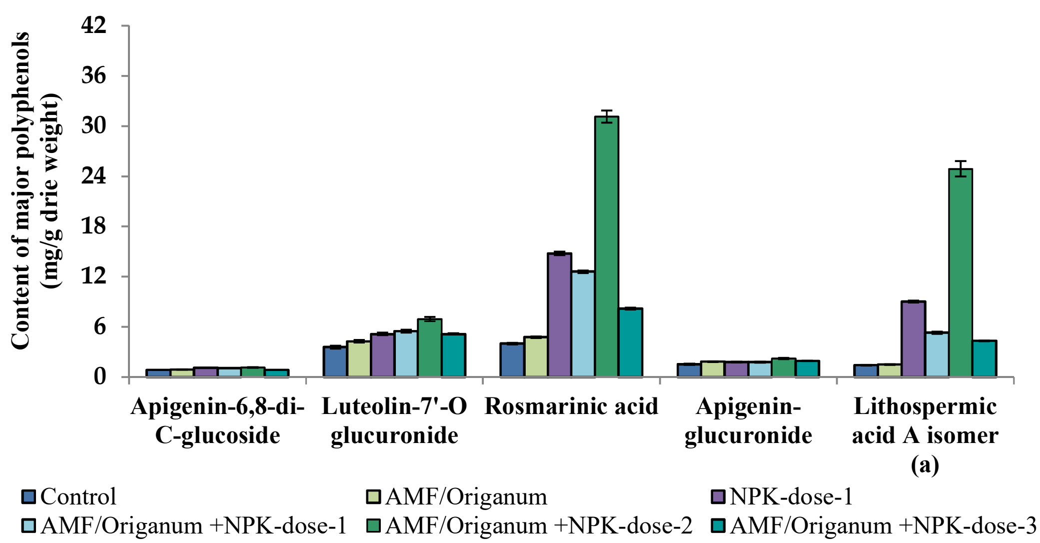


Fig.5: Polyphenol content of marjoram collected in September 2015. (AMF/Origanum-inoculum isolated from rhizosphere of marjoram, NPK-artificial fertilizer, dose-1-recommended does, dose-2 the half dose-3 third part of the recommended dose)

Lemon balm: Concerning the rosmarinic acid content there were no significant differences between the combined application of AMF inoculation and NPK-dodes-1,2,3 (treatment-3,4,5). There were also no significant differences between the positive control and the single

application of AMF inoculation and NPK (treatment-1,2), but these plants had higher rosmarinic acid content than the AMF+NPK treated ones (treatment-3,4,5).
The negative control plants had 2,5-fold higher luteolin-3-O-glucuronid content in comparison with the other treatments.

Marigold: Regarding the content of narcissin (one of the main components of marigold) the treatment-4 (AMF+NPK-dose2) was the most effective treatment similarly to marjoram. There were no significant differences between the other treatments. In the case of isorhamnetin-3-*O*-rhamnosyl-rutinoside (other main component of marigold) beside the combined application of AMF+ NPK-dose2 (treatment-4) the addition of NPK-dose3 (treatment-5) and the single AMF inoculation (treatment-1) were also sufficient.

**Conclusion**

The results indicate that a proper combination of AMF inoculum and an optimized dose of artificial fertilizer can increase the quality and the yield of medicinal plants.

## FORTH YEAR

### Aims and design

In 2016 the effect of two different commercially available AMF inocula was studied. For this purpose an open field and a semi-open field experiment were performed. During the semi-open field experiments we examined the absolute effect of the directed colonization.
In this year only marjoram has been planted, which gave the strongest response to AMF treatment during the previous years.
Unfortunately the composition of the INOQ-top inoculum, which was used during the previous years, has been modified by the producer. The new product under the same name contains only one AMF species (namely *Rhizophagusintraradices*) instead of the three species. In this year the efficiency of this mono-AMF inoculum was compared with another commercially available AMF inoculum called Symbivit, which is a mixture of several AMFspecies (*Claroideoglomus . etunicatum, G. microaggregatum, R. intraradices, C. claroideum, Funneliformis. mosseae, and F. geosporum*). In the open field experiment the effect of this two AMF product was compared with the most effective NPK dose, based on the results of the previous year. In the case of marjoram this dose was the half of the recommended NPK portion (NPK-dose-2 from 2015: N 25 kg/ha, P 35 kg/ha, K 65 kg/ha).
In the ***open field experiment*** the following six treatments were accomplished in three replications:

***Control***: soil of the exp. field
***Treatment-1***: soil of the exp. field + NPK-dose-2
***Treatment-2***: soil of the exp. field + AMF/INOQ
***Treatment-3***: soil of the exp. field + AMF/Symbivit
***Treatment-4***: soil of the exp. field + AMF/INOQ + NPK-dose-2
***Treatment-5***: soil of the exp. field +AMF/Symbivit + NPK-dose-2

In the ***semi-open field experiment*** the plants were planted into pots (5L) field with sterile mixture of the soil of the exp. field and potting soil. Sixteen pots were prepared and placed at the experiment field (sunken into the soil) in the case of the following three treatments:

***Control:*** sterile soil mixture
***Treatment-1***: sterile soil mixture + AMF/Symbivit
***Treatment-2***: sterile soil mixture + AMF/INOQ

**Main results of the open field experiment**

***Colonization***
In this year the intensity of the mycorrhizal infection was lower (M=15.5-24.8 %) than in the previous years. Inoculation with INOQ resulted higher mycorrhization level (M=24.5%) compared with Symbivit (M=15,5 %). Similarly to the observation of the previous year the application of the reduced dose of NPK did not supressed the colonization (M= 20.9-24.8 %). Control plants were colonized with native AMF populations found naturally in the soil.

***Biomass***
Despite of the lower colonization the use of Symbivit resulted the highest biomass. It was the double of AMF/INOQ treated plants' biomass. Interestingly the use of NPK reduced the biomass in all cases.

***Polyphenol content***
In this year beside rosmarinic acid luteolin-7'-*O*-glucuronide was the other main component instead of lithospermic acid isomer. Moreover the luteolin-glucoronid content was higher that the rosmarinic acid content in the herbal drug collected in August. In this year the polyphenol content was very low compared with the previous years. No significant differences were detected in the content of the main components between AMF/INOQ and AMF/Symbivit inoculated plants. Contrary to the result of biomass the highest polyphenol content was detected in NPK treated plants (treatment-1) in August. While in one month latter the control plants were the richest in polyphenol.

**Main results of the semi-open field experiment**

***Colonization***
The mycorrhizal colonization showed similarity to the results of the open-field experiment. Thus higher rate of colonization was observed in the case of AMF/INOQ (M=18.8%) compared with AMF/Symbivit (M=11.5).

***Biomass***
The effect of inoculation on the biomass yield was also similar to the results of open field experiment. The treatment with Symbivit inoculum was more productive than with INOQ.

***Polyphenol content***
The two main phenolic compounds were rosmarinic acid and lithospermic acid similar to the previous years. There were no significant differences between the control and AMF/INOQ treatments. The plants inoculated with Symbivit had significant lower phenolic acid content.

Interestingly the polyphenol content of plants from this semi-open field experiment was more than twenty times higher than from the open-field experiment.

**Conclusion**

There are big differences between the commercially available AMF inocula. Despite of the higher colonization intensity in the case of plants inoculated with Symbivit, the yield of them was higher in comparison with plants inoculated with INOQ-top. This finding indicates that beside the AMF species Symbivit contains other constituents which are more favourable for the growth of herbs. The results of the open and the semi-open field experiments show that the conditions of the cultivation (e.g. the quality of the soil) is significant in the case of the polyphenol profile and content of marjoram.

## FINAL OUTCOME OF THE STUDY

Benefits of AMF colonization on biomass and polyphenol production has been proved. Results of the present study show that AMF can provide different services for each herb. It shows that the effect and outcome of mycorrhization depends on the plant and also the fungal species. It was shown that the accumulation and alternation of different polyphenols in herb as the result of mycorrhizal colonization is a species specific mechanism. The results suggest that the application of optimized AM fungal inoculum in the cultivation of medicinal plants can be a great potential to improve the quantity and quality of the raw material. Thus AMF as an ecosystem service can play a key role in sustainable agriculture.

Beside the uploaded publications three thesis have been prepared (Corvinus University of Budapest, College of Károly Róbert). Moreover further publications are under preparation.

## FURTHER AIMS

We plan to identify the native AMF species found in the soil with molecular examinations. We would like to test the effect of mycorrhization on the quality of other medicinal plants. Moreover we plan to select further AMF species with positive effect on the production of active substances. Our further aims to optimize the mixture of the most beneficial AMF species, which can be used as inoculum in the cultivation of a large scale of herbs.

One of our further aims is to design a longitudinal research in order to have more information on the role of the AMF in the accumulation of active ingredients in herbs and to understand the background mechanisms.

## MAIN CHANGES IN THE BUDGET

The duration of the project was expanded with one year. In this additional year further open field experiments have been accomplished combined with laboratory work. For this reason the remained amount of the budget has been redistributed and László Grezner was employed in part-time for seasonal tasks related to the maintenance of the experimental field and extra allowance was paid to Krisztina Szabó (an employee of the institute) for her work in the field (planting, sampling, data collection, harvesting etc.) and in the laboratory (sample preparation). Personal changes was only in the employment of field worker.

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## REFERENCES

1. Brundrett, M. C. Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil. 2009, 320, 37-77.
2. Marschner, H. The soil-root interface (rhizosphere) in relation to mineral nutrition In Mineral nutrition of higher plants, 2 ed.; Marschner, H., Ed. Academic Press: London, 1997; pp 537-594.
3. Parniske, M. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. 2008, 6, 763-775.
4. Heijden, M. G. A.; Scheublin, T. R.; Brader, A. Taxonomic and functional diversity in arbuscular mycorrhizal fungi - is there any relationship? New Phytologist 2004, 164, 201-204.
5. Gianinazzi, S.; Gollotte, A.; Binet, M.-N.; van Tuinen, D.; Redecker, D.; Wipf, D. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 2010, 20, 519-530.
6. Gosling, P.; Hodge, A.; Goodlass, G.; Bending, G. D. Arbuscular mycorrhizal fungi and organic farming. Agric. Ecosyst. Environ. 2006, 113, 17-35.
7. Takács, T. Site-specific optimization of arbuscular mycorrhizal fungi mediated phytoremediation. In Toxicity of heavy metals to legumes and bioremediation 1ed.; Zaidi, A.; Ahmad, W. P.; Khan, M. S., Eds. Springer-Verlag: Berlin Heidelberg, 2012; pp 179-202.
8. Zeng, Y.; Guo, L. P.; Chen, B. D.; Hao, Z. P.; Wang, J. Y.; Huang, L. Q.; Yang, G.; Cui, X. M.; Yang, L.; Wu, Z. X.; Chen, M. L.; Zhang, Y. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 2013, 23, 253-265.
9. Chen, Y. L.; Li, J. X.; Guo, L. P.; He, X. H.; Huang, L. Q. Application of AM Fungi to Improve the Value of Medicinal Plants. In Mycorrhizal Fungi: Use in Sustainable Agriculture and Land Restoration, Solaiman, Z.; Abbott, L. K.; Varma, A., Eds. Springer-Verlag: Berlin Heidelberg, Germany, 2014; pp 171-187.
10. Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. Nat. Prod. Rep. 2009, 26, 1001-1043.
11. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 2004, 79, 727-747.
12. Scalbert, A.; Manach, C.; Morand, C.; Remesy, C.; Jimenez, L. Dietary polyphenols and the prevention of diseases. Crit. Rev. Food. Sci. Nutr. 2005, 45, 287-306.
13. Okuda, T. Antioxidants in Herbs: Polyphenols. In Antioxidant Food Supplements in Human Health, Packer, L.; Hiramatsu, M.; Yoshikawa, T., Eds. Academic Press: California, USA, 1999; pp 393-410.
14. Mieseler, T. Calendula An Herb Society of America Guide. http://www.herbsociety.org (22/01/2016).

15. Meyers, M. Oregano and Marjoram An Herb Society of America Guide to the Genus Origanum. http://www.herbsociety.org (22/01/2016).
16. Meyers, M. Lemon Balm: An Herb Society of America Guide. http://www.herbsociety.org (22/01/2016).
17. Trouvelot, A.; Kought, J. l. Mesure du taux de mycorhization VA d'un systéme radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In Physiological and genetical aspects of mycorrhizae, Gianinazzi-Pearson, V.; Gianinazzi, S., Eds. INRA: Paris, France, 1986; pp 217-221.
18. Abranko, L.; Garcia-Reyes, J. F.; Molina-Diaz, A. Systematic bottom-up approach for flavonoid derivative screening in plant material using liquid chromatography high-resolution mass spectrometry. Anal. Bioanal. Chem. 2012, 403, 995-1006.
19. Engel R, Szabó K, Abrankó L, Rendes K, Füzy A, Takács T: Effect of Arbuscular Mycorrhizal Fungi on the Growth and Polyphenol Profile of Marjoram, Lemon Balm, and Marigold, J. Agr. Food Chem. 2016. 64: (19) 3733-3742
20. Engel R, Szabó K, Abrankó L, Sárosi Sz, Anna Füzy, Tünde Takács: Effect of fertilization and arbuscular mycorrhizal fungi on active substances of marjoram, Planta Medica, 81: PW_144, 63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 2015. 08. 23-27
21. Engel R, Szabó K, Abrankó L, Füzy A, Takács T: Effect of arbuscular mycorrhizal fungi and fertilization on polyphenol profiles of marjoram, lemon balm and marigold, Book of abstracts, 7th International Conference on Polyphenols and Health, Tours, France, 2015. 10. 27-30, p.295.
22. Engel R, Szabó K, Abrankó L, Füzy A, Takács T: Különböző eredetű arbuszkuláris mikorrhiza gombák hatása a majoránna, a citromfű és a körömvirág hatóanyag - termelésére, In: Padisák Judit, Liker András, Stenger-Kovács Csilla (szerk.) (szerk.) X. Magyar Ökológus Kongresszus. Veszprém, Hungary, 2015.08.12-2015.08.14. pp. 50,