Effect of Arbuscular Mycorrhizal Fungi on the Growth and the Polyphenol Profile of Marjoram, Lemon Balm and Marigold

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ABSTRACT

The aim of this study is to examine the effect of arbuscular mycorrhizal fungi (AMF) colonization on biomass, polyphenol profile and content of economically important herbs. A pot experiment was performed with marjoram, lemon balm and marigold applying a commercially available AMF mixture for inoculation. Major polyphenols were identified using HPLC-UV-ESI-qTOFMS based on their UV-Vis and mass spectral characteristics and selected ones were quantified. We showed that AMF can provide different services for each herb. Marjoram had the highest level of fungal colonization (82 M%) followed by lemon balm (62 M%) and marigold (17 M%). AMF inoculation significantly increased the biomass of marjoram (1.5 fold), the number of marigold's flowers (1.2 fold) and the yield of rosmarinic acid and lithospermic acid isomers of marjoram (1.5 fold) and lemon balm (1.2 fold). Therefore the quantity and quality of plant material could be improved by the application of optimized AMF inoculum.

KEYWORDS

Origanum majorana, *Melissa officinalis*, *Calendula officinalis*, arbuscular mycorrhizal fungi, polyphenol

INTRODUCTION

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 [1]. For instance, polyphenol rich diet can play an important role in the prevention of various diseases such as cardiovascular and neurodegenerative disorders [2, 3]. Besides fruits and vegetables, many herbs and spices serve as excellent sources of phenolic compounds [4], 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 [5-7].

Calendula officinalis L. is an annual plant with yellow or orange flowers, which is consumed as tea, used for coloring and flavoring food and chopped into salads. The dried flowers have a long tradition of medicinal use due to their numerous biological activities including anti-inflammatory, anti-septic, antibacterial, antifungal, wound healing, antioxidant and antispasmodic effects. Infusions, ointments and tinctures made from the flowers are frequently used for healing wounds, bruises and burns. Furthermore marigold preparations can be used to treat dermatitis, ulcers, herpes and abdominal cramps. The main phytochemical constituents related to the positive effects of marigold are essential oils, triterpenoids, carotenoids, flavonoids (glycosides of isorhamnetin and quercetin) and phenolic acids [5, 8-10].

53 *Origanum majorana* L. is a popular and commercially important spice, as it gives the flavor of a
54 large scale of dishes and it is an essential ingredient in various processed foods. Besides its
55 culinary value, sweet marjoram is used in traditional medicine owing to its carminative,
56 antispasmodic, diaphoretic, diuretic, antioxidant, antimicrobial and anti-inflammatory
57 attributes. Different preparations and extracts of its flowering shoots can be applied to treat
58 gastrointestinal disturbances, cough, bronchical diseases. It is also used in mouthwashes for
59 hygiene. The biologically active ingredients of this herb are essential oils, flavonoid glycosides,
60 phenolic acids, tannins and triterpenoids [6, 11, 12].
61 *Melissa officinalis* L. is also a versatile culinary herb which can be used to flavor many different
62 types of dishes from appetizers to desserts. One of the most preferred ways to consume is tea.
63 The leaf of lemon balm is commonly used medicinally to treat nervous disturbances (anxiety,
64 insomnia, stress) and gastrointestinal disorders. Its numerous beneficial effects, such as
65 sedative, spasmolytic, antimicrobial, antioxidant, antitumoral actions are attributed to the
66 presence of flavonoids, phenolic acids, triterpenoids and tannins [7-9, 13, 14].
67 Pot marigold, sweet marjoram and lemon balm used in the food and pharmaceutical industry
68 are mainly gained from cultivation where several growing conditions can be controlled. With
69 the development of new agricultural techniques, the yield and quality of plant material can be
70 improved significantly to fulfill the increasing industrial and consumer demands. One of the
71 promising possibilities for enhancing the biomass and increase the quality of herbs can be the
72 application of arbuscular mycorrhizal fungi (AMF) during their cultivation. AMF occur in all
73 terrestrial ecosystems and form symbiotic associations with the majority of terrestrial plant
74 species, including many agriculturally and horticulturally important crop species [15]. 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 [16]. These fungi are obligate biotrophs, which must develop a symbiotic association with the host in order to grow and complete their life cycle [17]. 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 [18]. 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 [19-21]. 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 [19, 22, 23]. However, the way of this influence on the active substances is not always evident. The presence of AMF might either enhance the accumulation of the secondary metabolites in the plant's organs but in some cases there is no detectable effect of it. Recent observations indicate that the influence of the AMF colonization on the level of secondary metabolites highly depends on the type of host plant and the fungi species. For example, the polyphenol content of *Ocimum basilicum* L. was increased as a result of the colonization by *Glomus caledonium*, while the colonization by *Glomus intraradices* had no effect [24]. At the same

time, the inoculation with *Glomus intraradices* increased the polyphenol content of *Echinacea purpurea* (L.) Moench. [25]. Consequently, the use of proper AMF during the cultivation can improve the quantity as well as the quality of the obtained plant material.

Although the chemical composition and pharmacological activities of pot marigold, sweet marjoram and lemon balm have been well studied and earlier findings show that these herbs colonized by AMF, no data are available about the influence of AMF on the polyphenol content of these important plant species. Thus, the aim of the present study is to examine the effect of a commercially available AMF mixture on the profile and content of phenolic compounds of pot marigold, sweet marjoram and lemon balm. Our findings allow us to evaluate the possibility of eco-friendly agricultural application of AMF in order to improve the cultivation of selected herbs.

MATERIALS AND METHODS

Plant Material and Experimental Design. Pot marigold, sweet marjoram and lemon balm were chosen in this study since these species favorably 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 drugs, i.e. the parts rich in active substances. A pot experiment was performed in plant-growth room involving marigold, marjoram and lemon balm grown with and without AM fungal inoculation. In order to ensure the maximal

119 homogeneity of the plant material, three reliable and commercially available cultivars were
120 chosen for this study: *Calendula officinalis* 'Calypso Orange with Black Eye', *Melissa officinalis*
121 'Relax', *Origanum majorana* 'Esperanta'. The seeds were purchased from Volmary GmbH
122 (Münster, Germany). Pre-germinated seeds were transplanted to pots (1200 mL growth
123 medium/pot). One plant of marigold, four plants of marjoram and four plants of lemon balm
124 were grown per pot. Mycorrhizal and non-mycorrhizal control plants were grown on pumice
125 media for 15 weeks under controlled conditions (8h/16h-25000lux; 17°C/27°C day/night,
126 relative humidity 50-70%). Soil-analog pumice medium is an AMF free and a chemically inert
127 porous volcanic rock with 0.6–1 mm particle size, pH of 6.5, 0.94 kg/L bulk density and 0.26
128 cm^3/cm^3 water content at field capacity. Plants were watered with tap water as required and
129 fertilized with 120 mL of P poor Hoagland nutrient solution (0.1 mM KH_2PO_4) once a week. For
130 the AMF treatment 24 g/pot INOQ commercially available inoculum (INOQ GmbH, Schnega,
131 Germany) was added to the top third part of pumice media before plantation to promote the
132 early contact between AMF and the young roots. Mycorrhizal plants were grown under the
133 same conditions as control plants. In the case of each plant species, 24 pots were prepared in
134 total. Twelve pots of AMF treated and 12 pots of non-treated plants were placed randomly (not
135 separately) in growth chamber. The commercial AMF inoculum was a mixture of
136 *Claroideoglomus etunicatum* (Schüssler and Walker), *Claroideoglomus claroideum* (Schüssler
137 and Walker) and *Rhizophagus intraradices* (Schüssler and Walker).
138 **Harvesting.** The widely used parts of the three plant species such as flowers of marigold,
139 flowering shoots of marjoram, leafy shoots (top 10-15 cm) of lemon balm were collected from
140 the sixth week after plantation. All pots were monitored weekly and new flowers, flowering

shoots and leafy shoots were collected from each pot if there were any. All samples collected from the same pot were pooled. For the analytical measurements pools were formed also from the AMF-treated and non-treated samples. Fresh and dry weights of collected plant material were weighed. Parts of the plants were dried at 40°C in drying oven. Diameters of the flowers of marigold were measured, number of flowers per pot was counted. After drying, 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 materials were used for chemical analyses.

Root Colonization. Root samples of control and mycorrhizal plants were taken at the middle (3rd week, 3 replicates) and at the end of the vegetation period (15th week, 9 replicates). Root samples of the three herbs were cleaned and stained by cotton blue (1 w/v%) solution [26, 27]. Mycorrhizal colonization was estimated by examining 1 cm-root segments (30 segments/root sample) under dissecting microscope (40-60X; Olympus B71). AM fungal colonization was expressed as mycorrhizal frequency (F%), intensity of colonization (M%) and arbuscule richness (A%) [28].

Chemicals and Reagents. All reagents were of analytical grade. Acetonitrile, methanol (LC-MS grade) and formic acid (~98%) were purchased from Merck (Merck, Darmstadt, Germany). A Milli-Q ultrapure water system (Merck Millipore, Billerica, MA, USA) was used throughout the study to obtain high purity water for the HPLC (high performance liquid chromatography) analysis. Crystalline reference substances of luteolin 7-glucoronide, lithospermic acid A, isorhamnetin 3-rutinoside, isorhamnetin 3-neohesperidose, rutin, apigenin, rosmarinic acid, chlorogenic acid were purchased from Phytolab GmbH (Vestenbergsgreuth, Germany).

Sample Preparation. Sample preparation procedure was adopted from Abranko et al. [29] and applied with slight modifications. An amount of 200 mg dried and ground plant material from the pools of samples was weighed into a centrifuge tube (50mL), and 10 mL 60% aqueous MeOH containing 1% formic acid was added. Samples were extracted for 1 hour in an ultrasonic bath (<35 °C at the end). Extracts were centrifuged for 10 min at 8000 g. An aliquot of 2.5 mL supernatant was transferred into a 15-mL graded centrifuge tube and diluted to 10 mL with water. Finally, it was filtered (Cronus 25 mm PTFE Syringe Filter 0.22µm) before injecting to the HPLC. In case of each sample, six replicates were extracted and analyzed separately.

Instrumentation. 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 Agilent (Santa Clara, CA USA) 6530 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 on a Waters Alliance HPLC-system equipped with photodiode array detector (PDA) together with a quaternary pump, an auto-sample injector, an on-line degasser and an automatic thermostatic column oven (Waters Corp., Milford, MA, USA). The same chromatographic conditions (analytical column, mobile phases, gradient program) were applied in both systems (described in detail below).

HPLC-ESI-qTOFMS analysis. The q-TOFMS was used with the following operation parameters: capillary voltage, ±4,000 V; nebulizer pressure, 40 psig; drying gas flow rate, 13 l/min; gas temperature, 350 °C. During these experiments, fragmentor voltage was triggered automatically between 160 V and 210 V in positive mode and 140 and 240 V in negative mode. The lower value represents mild conditions in order to minimize in-source fragmentation, while

185 the higher one is to foster in-source fragmentation. Full-scan mass spectra in the range of *m/z*
186 50-1100 were recorded at 1.5 spectra/s scanning speed at all times during the chromatographic
187 run. The instrument performed the internal mass calibration automatically, using an automated
188 calibrant delivery system, which introduces the flow from the outlet of the chromatograph
189 together with a low-flow (approximately 10 µL/min) of a calibrating solution. The UV-Vis
190 detector was acquiring data in the range of 200-800 nm in 2-nm steps at 0.5 spectra/s
191 acquisition speed [29].
192 **Chromatographic Conditions.** Chromatographic separation was carried out on a Phenomenex
193 Kinetex Phenyl-hexyl, 4.6×150 mm, 2.6 µm column (Torrance, CA, USA). For elution, 0.1% (v/v)
194 formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B)
195 were used as solvents at a flow rate of 500 µL/min. The gradient program started at 10% B, and
196 after 5 min of isocratic run, solvent B was increased linearly and reached 45% at 35 min and
197 then 100% at 40 min. Finally, 100% B was kept constant for 5 min before getting back to initial
198 conditions to equilibrate the LC before the next injection. Detection wavelength was 330 nm,
199 which was chosen as a common wavelength according to the UV-Vis spectra of the identified
200 compounds (**Table 1-3**). The sample injection volume was 5 µL for the identification and 10 µL
201 for the quantitative determination of polyphenols.
202 **Quantification.** Identified compounds were quantified based on their UV-Vis signals using
203 reference standards. Luteolin 7-glucoronide, lithospermic acid A, isorhamnetin 3-rutinoside
204 (narcissin), isorhamnetin 3-neohesperidose (calendoflavoside), rutin, rosmarinic acid and
205 chlorogenic acid were quantified based on a multi-point (5, 10, 20, 50, 100 µg/mL) calibration at
206 330 nm using reference standards. In the cases of those compounds, where reference

substances were not available, calibration functions of similar available compounds were employed to estimate their concentrations. Particularly, both lithospermic acid A isomers were quantified based on the calibration function of lithospermic acid A, whereas apigenin-6,8-di-*C*-glucoside and apigenin-glucuronide were quantified using the calibration function for apigenin aglycone. Moreover, quercetin-3-*O*-rhamnosyl-rutinoside was quantified using the calibration function of rutin, whereas the calibration function of isorhamnetin-3-*O*-rutinoside was used to estimate the concentration of isorhamnetin-3-*O*-rhamnosyl-rutinoside and isorhamnetin-malonyl-glucoside. A correction factor derived from the quotient of the molecular weight of the compound used for calibration and that of the unknown one was applied in all cases to be able to express results in mg/g dry weight [30].

Statistical analysis. Data of yield and polyphenol content were analyzed by non-parametric Mann-Whitney U test ($p \leq 0.05$) using StatSoft's STATISTICA version 10.

RESULTS

Identification of phenolic compounds. Figure 1. (**a-c**) shows the phenolic compound profiles of marjoram, lemon balm and marigold, respectively. Phenolic compounds were tentatively identified based on UV spectra and accurate mass fragmentation information [12, 13, 29-32]. Retention time, UV-Vis and mass spectral characteristics, as well as tentative identification of compounds are presented in **Table 1-3**. When standard substances were available, retention time information was also used for compound confirmation. These compounds are labelled with asterisk in **Table 1-3**. As shown in **Figure 1**. **(a-c)**, most compounds with prevalent UV

signal at 330 nm, a characteristic wavelengths of polyphenols having a subunit with a cinnamoyl structure, were tentatively identified. In particular, seven phenolic compounds in marjoram, six in lemon balm and eight in marigold. Among them 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.

AMF Root Colonization. In the microbial treatment all three herb species formed symbiotic relations with the added commercial AMF inoculum. No AMF colonization was detected in control groups (**Table 4**.). The root system of the three plant species were colonized in different rates. According to the root colonization parameters shown in **Table 4**., marjoram plants had the highest level of fungal colonization followed by lemon balm and marigold. The AMF root colonization in marjoram reached almost 100% after 15 weeks of growth, and the arbuscule density (A%) was also very high. In lemon balm, the AMF root colonization can be characterized by high intensity of colonization but low arbuscule richness. The intensity of colonization and the arbuscule density in marigold were very low compared to the other two species (**Table 4**.).

Yield. 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 (**Table 4**.).

Polyphenols. Quantitative data of main phenolic compounds of marjoram, lemon balm and marigold are summarized in **Table 5-6**. In terms of polyphenol content, the effect of mycorrhization was different in the three examined herbs. In the case of marjoram, a significant decrease of three active substances was observed in plants colonized by AMF (**Table 5**.). In these plants, the two main phenolic compounds, namely rosmarinic acid and lithospermic acid A isomer were presented in 28 and 24 % lower concentration than in the non-mycorrhizal control plants. The highest level of decrease (50%) was detected in apigenin-6,8-di-*C*-glucoside content. Regardless of the lower concentration of the above mentioned compounds, mycorrhization increased the total yield of polyphenols calculating on the yield of biomass (**Table 5**.). The total yield of polyphenols was calculated by multiplying concentration by the biomass dry weight. **Figure 2.** demonstrates the alternation between the content and yield of polyphenols through the example of rosmarinic acid. With regard to lemon balm, the mycorrhization also influenced the concentration of phenolic acids, but partially in a different manner. 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 (**Table 6**.). Regarding 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 (**Table 7**.)

DISCUSSION

The health promoting properties of marjoram, lemon balm and marigold, besides other group of active substances such as essential oil, can be attributed to their polyphenol content. The polyphenol profiles of examined species showed similarity to previous findings. In agreement with the literature data, rosmarinic acid was the main constituent of marjoram and lemon balm [11, 13]. We also identified lithospermic acid A isomers in these *Lamiaceae* species, one from marjoram and two from lemon balm [12, 13]. In marjoram, besides the lithospermic acid A isomer, litospermic acid B (also known as salvianolic acid B) a dimer of rosmarinic acid was also present in accordance with the recent findings of Damašiu et al. [31]. Luteolin-7'-*O*-glucuronide was the most abundant flavonoid in marjoram samples, whereas luteolin-3'-*O*-glucuronide was not detectable in our samples, despite the fact that it was found as the main flavonoid of lemon balm in other studies [13, 33] . In lemon balm samples, besides caftaric acid hexosid, caftaric acid was also present in traces. The polyphenol profile of the examined marigold samples was in a good agreement with that of Alves Fernandes et al [32]. According to their results, narcissin and isorhamnetin-3-*O*-rhamnosyl-rutinoside were the main compounds of marigold. Above the seven compounds described by Alves Fernandes et al. [32] isorhamnetin-malonyl-glucoside was also found in our samples. These differences can be attributed to the chemical diversity of breeds and also to the different growing conditions.

In the terms of mycorrhizal status, marjoram, lemon balm and marigold belong to herbs forming symbiotic association with arbuscular mycorrhizal fungi. Earlier investigations also indicated that these plant species are colonized by a wide range of AMF [34-40]. Our results show that mycorrhization was significantly influenced by plant species. Abundance of AMF in roots

294 was significantly different among plants species (**Table 4**). Marjoram had the most positive
295 reaction to inoculation with commercial AMF mixture (M%: 82) compared to lemon balm (M%:
296 62) and marigold (M%: 17). The values of mycorrhizal parameters in marigold were the lowest
297 of all three herbs. These findings are consistent with previously reported data. Despite the high
298 variation in root colonization parameters, in general, the same robust tendency in the
299 mycorrhizal status of these three plants is presented by the literature. Thus the colonization
300 level is the lowest of marigold (22-59%) [34, 37, 40], while lemon balm (12-100%) [36, 39, 40] and
301 marjoram (24-97%) [35, 38-40] can be characterized with high AMF colonization affinity.
302 The high abundance of mycotroph plant species and high level of AMF colonization in roots
303 indicate that AMF play an important role in natural ecosystems and agroecosystems, providing
304 a range of benefits to the host plants. One of these benefits is the improved uptake of
305 nutrients, which can be manifested in the enhanced growth and stress tolerance of the host
306 plants. Our study showed also a significant growth increase of marjoram when colonized with
307 AMF. This result is in accordance with a number of studies related with AMF-herb associations.
308 Enhanced biomass production was observed due to AMF colonization in *Ocimum basilicum* L. [41],
309 *Echinacea purpurea* L. [25], *Catharanthus roseus* L. [42] and *Salvia officinalis* L. [43]. Thus AMF
310 inoculation of marjoram plants results in positive impact on biomass. A plausible explanation of
311 increased plant growth with AMF colonization might be related to higher nutrient uptake
312 efficiency. Contrary to the result of marjoram, AMF inoculation increased neither the biomass
313 of lemon balm nor the yield of marigold flowers. Although increased number of flowers was
314 observed due to the AMF colonization, these marigold flowers were smaller, thus there were
315 no significant difference in the flower yield compared to control plants. Our findings are in

316 agreement with the results of Zaller et al. [34], but in contradiction with the study of Tabrizi et al.
317 [37]. According to the results of Zaller et al. [34] the number of flower buds significantly increased
318 by inoculation with AMF mixture of four *Glomus* taxa, but the total biomass remained
319 unaffected. On the contrary, Tabrizi et al. [37] observed that the mycorrhization by *Glomus*
320 *mosseae* and *G. intraradices* significantly improved the flower yield of marigold. Our findings
321 related to the biomass of lemon balm show similarity with those studies where no change was
322 observed in the biomass in the presence of *Glomus* taxa in *Ocimum basilicum* L. another
323 *Lamiaceae* species [24, 44].
324 In addition to its effect on growth, mycorrhization can also influence the levels of secondary
325 metabolites in medicinal plants [24]. Several groups of secondary metabolites with different
326 functions are produced during the development of herbs. Studies related to AMF-medicinal
327 plants associations mainly focused on the effect of AM symbiosis on essential oil content. Most
328 of these reports confirmed that essential oil content of medicinal plants – such as basil, mint,
329 oregano and dill – increased due to the AMF inoculation [22, 41]. Evidence shows that AMF can
330 stimulate the production of alkaloids e.g. in *Catharanthus roseus* L. [22, 42] and hypericin in
331 *Hypericum perforatum* L. [22].
332 However, only a few investigations have focused on the effects of AMF on the accumulation of
333 polyphenols in aerial parts of herbs. Some reports have given information on how
334 mycorrhization influences phenolic compounds in the roots of host plants. Larose et al. [45] found
335 that flavonoid accumulation in *Medicago sativa* roots depends on the root-colonizing AMF. In
336 their experiments coumesterol, daidzein, genistein and biochanin A were accumulated in all
337 mycorrhizal plants, but e.g. coumesterol was presented in higher concentration in roots

colonized *by Glomus intraradices* and *Gigaspora rosea* than in roots colonized by *G. mosseae* [45]. In case of *Echinacea purpurea* L., colonization with *G. intraradices* significantly increased the content of phenolics in the root, especially of cynarin and cichoric, caftaric and chlorogenic acid [25]. Mycorrhization was also related to increased concentration of total phenolic acids in the root of *Arnica montana* L. [46]. Furthermore, in the root of *Trifolim repens* inoculated with *Glomus intraradices* quercetin, acacetin and rhamnetin were detected, which were not presented in the root of non-inoculated plants [47].

To the best of our knowledge this is the first time when the effect of mycorrhization was investigated on the polyphenol profile of the aerial parts of marjoram, lemon balm and marigold. According to our results, inoculation with AMF mixture did not change the composition of the main polyphenols, but concentrations of certain compounds were influenced. It was shown that mycorrhizal symbiosis clearly increased the content and the total yield of rosmarinic acid and lithospermic acid A isomers in lemon balm. Our results also showed that mycorrhization not uniformly affects polyphenol content in the studied herbs. For instance, marjoram plants colonized by AMF showed a significant decrease in the content of rosmarinic acid, lithospermic acid isomer and apigenin-6,8-di-*C*-glucoside in comparison with non-inoculated plants. It should be noted that the decrease of polyphenol concentrations in combination with the improved plant growth resulted in an overall improved polyphenol yield in marjoram. Although the AMF inoculation did not cause any changes in the concentration and the yield of major polyphenols of marigold, it significantly increased the total yield of a minor component, namely isorhamnetin-malonyl-glucoside.

There are contradictory data in the literature about how AM symbiosis influences the polyphenol content in the aerial parts of medicinal plants. Hristozkova et al. [35] investigated the effect of inoculation with two strains of *Claroideoglomus claroideum* and a *Glomus mosseae* on the total phenol and flavonoid concentration in the aerial part of marjoram. They found a significant decrease in total phenol concentration in plants colonized by *G. mosseae*. While the total flavonoid concentration was higher in the plants inoculated by one of the *C. claroideum* strains compare with the other treatments. While *G. mosseae* did not affect the total flavonoid content of marjoram, enhancement of total flavonoid content was observed in the flowers of marigold plants colonized by *Glomus mossea* and *G. intraradices* [37]. In terms of AM symbiosis, various data can also be found related with *Ocimum basilicum* L. another *Lamiaceae* species in which also rosmarinic acid is one of the dominant phenolic acids. In an investigation of functional diversity among three *Glomus* species (*G. intraradices*, *G. caledonium*, *G. mosseae*) *Glomus caledonium* increased the rosmarinic acid content in the shoots of basil, while the others in this aspect remained ineffective [24]. In another study, similarly to the above mentioned, the inoculation with *G. intraradices* had no significant effect on polyphenolic content and profile of the leaves of basil, but increased the total anthocyanin concentration [44]. Enhancing effect of AMF-inoculation on the total phenolic content in the leaves of *Catharanthus roseus* and modifying effect on the polyphenol profile of artichoke have been reported [42, 48].

Our findings in accordance with literature data suggest that the mycorrhizal influence on the secondary metabolite pathways is a result of a complex mechanism depending on several circumstances such as the species of fungus and host plant, the rate of mycorrhization, nutrient

content of the soil, health condition of the plants, environmental factors, amongst others. There are several theories for the reasons of changes in polyphenol content induced by mycorrhization. One of the possible explanations for the increased phenolic acid concentration is related to improved nitrogen uptake. Since tyrosine and phenylalanine are the main precursors of rosmarinic acid, the higher nitrogen uptake in AM plants might have contributed to the increased production of these amino acids [24, 49]. As a possible option, AMF induced changes in phytohormone level in host plants could be in the background of this phenomenon [24]. Another reason for the positive and negative alternation of phenolic acid content in AM plants might be that the polyphenols can act as signaling and/or as regulatory compounds in plant-microbe symbioses [45, 50]. Nevertheless further in-depth research is required 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. The interaction of the host plants with the AMF depends on the genotype of the partners and on several environmental factors. In contrast to the large number of herbs that form AM, limited knowledge is still available about their mycorrhizal dependence and AMF preference.

Overall, this was the first time when the effect of mycorrhization on the polyphenol profile of marjoram, lemon balm and marigold was determined. Results of the present study show that AMF can provide different services for each herb. For instance while marjoram benefits more from the AMF colonization in terms of growth, lemon balm has higher content of phenolic acids. 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.

Our further aim is to set up an open field experiment in order to examine the effect of the AMF inoculation in the agricultural practice. Selection of compatible symbiotic partners and application of AM fungi adapted functionally to the site specific conditions can be a successful innovation for the future of plant cultivation.

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NOTES

The authors declare no competing financial interest.

REFERENCES

1. Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, 26, 1001-1043.
2. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, 79, 727-747.
3. 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.
4. 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.
5. Mieseler, T. Calendula An Herb Society of America Guide. http://www.herbsociety.org (22/01/2016).
6. Meyers, M. Oregano and Marjoram An Herb Society of America Guide to the Genus Origanum. http://www.herbsociety.org (22/01/2016).
7. Meyers, M. Lemon Balm: An Herb Society of America Guide. http://www.herbsociety.org (22/01/2016).
8. *WHO Monographs on Selected Medicinal Plants*. World Health Organization: Geneva, 1999; Vol. 2.
9. *European Pharmacopoeia 6th* ed. Council of Europe: Strasbourg France, 2007; Vol. 1.
10. Lim, T. K. Calendula officinalis. In Edible Medicinal And Non-Medicinal Plants, Flowers, Lim, T. K., Ed. Springer International Publishing AG: Cham, Switzerland, 2014; Vol. 7, pp 213-244.

443 11. Fecka, I.; Turek, S. Determination of polyphenolic compounds in commercial herbal drugs
444 and spices from Lamiaceae: thyme, wild thyme and sweet marjoram by chromatographic
445 techniques. *Food Chem*. **2008**, 108, 1039-1053.
446 12. Kaiser, A.; Carle, R.; Kammerer, D. R. Effects of blanching on polyphenol stability of
447 innovative paste-like parsley (Petroselinum crispum (Mill.) Nym ex A. W. Hill) and marjoram
448 (Origanum majorana L.) products. *Food Chem*. **2013**, 138, 1648-1656.
449 13. Barros, L.; Duenas, M.; Dias, M. I.; Sousa, M. J.; Santos-Buelga, C.; Ferreira, I. C. F. R.
450 Phenolic profiles of cultivated, in vitro cultured and commercial samples of Melissa
451 officinalis L. infusions. *Food Chem*. **2013**, 136, 1-8.
452 14. Fecka, I.; Turek, S. Determination of water-soluble polyphenolic compounds in commercial
453 herbal teas from Lamiaceae: Peppermint, melissa, and sage. *J. Agric. Food Chem.* **2007**, 55,
454 10908-10917.
455 15. Brundrett, M. C. Mycorrhizal associations and other means of nutrition of vascular plants:
456 understanding the global diversity of host plants by resolving conflicting information and
457 developing reliable means of diagnosis. *Plant Soil.* **2009**, 320, 37-77.
458 16. Marschner, H. The soil-root interface (rhizosphere) in relation to mineral nutrition In
459 Mineral nutrition of higher plants, 2 ed.; Marschner, H., Ed. Academic Press: London, 1997;
460 pp 537-594.
461 17. Parniske, M. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev.*
462 *Microbiol*. **2008**, 6, 763-775.

18. 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.

19. 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.

20. Gosling, P.; Hodge, A.; Goodlass, G.; Bending, G. D. Arbuscular mycorrhizal fungi and organic farming. *Agric. Ecosyst. Environ*. **2006**, 113, 17-35.

21. 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.

22. 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.

23. 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.

24. Toussaint, J. P.; Smith, F. A.; Smith, S. E. Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza* **2007**, 17, 291-297.

485 25. Araim, G.; Saleem, A.; Arnason, J. T.; Charest, C. Root colonization by an arbuscular
486 mycorrhizal (AM) fungus increases growth and secondary metabolism of purple
487 coneflower, Echinacea purpurea (L.) Moench. *J. Agric. Food Chem*. **2009**, 57, 2255-2258.
488 26. Phillips, J. M.; Hayman, D. S. Improved procedures for clearing roots and staining parasitic
489 and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br.*
490 *Mycol. Soc.* **1970**, 55, 158-161.
491 27. Grace, C.; Stribley, D. P. A safer procedure for routine staining of vesicular-arbuscular
492 mycorrhizal fungi. *Mycol. Res.* **1991**, 95, 1160-1162.
493 28. Trouvelot, A.; Kought, J. l. Mesure du taux de mycorhization VA d'un systéme radiculaire.
494 Recherche de méthodes d'estimation ayant une signification fonctionnelle. In Physiological
495 and genetical aspects of mycorrhizae, Gianinazzi-Pearson, V.; Gianinazzi, S., Eds. INRA:
496 Paris, France, 1986; pp 217-221.
497 29. Abranko, L.; Garcia-Reyes, J. F.; Molina-Diaz, A. Systematic bottom-up approach for
498 flavonoid derivative screening in plant material using liquid chromatography high-
499 resolution mass spectrometry. *Anal. Bioanal. Chem*. **2012**, 403, 995-1006.
500 30. Lin, L.-Z.; Harnly, J.; Zhang, R.-W.; Fan, X.-E.; Chen, H.-J. Quantitation of the
501 Hydroxycinnamic Acid Derivatives and the Glycosides of Flavonols and Flavones by UV
502 Absorbance after Identification by LC-MS. *J. Agric. Food Chem*. **2012**, 60, 544-553.
503 31. Damasius, J.; Venskutonis, P. R.; Kaskoniene, V.; Maruska, A. Fast screening of the main
504 phenolic acids with antioxidant properties in common spices using on-line HPLC/UV/DPPH
505 radical scavenging assay. *Anal. Methods*. **2014**, 6, 2774-2779.

506 32. Alves Fernandes, E. F.; Meloni, F.; Borella, J. C.; Lopes, N. P. Effect of fertilisation and harvest
507 period on polar metabolites of Calendula oficcinalis. *Rev. Bras. Farmacogn*. **2013**, 23, 731-
508 735.
509 33. Heitz, A.; Carnat, A.; Fraisse, D.; Carnat, A. P.; Lamaison, J. L. Luteolin 3 '-glucuronide, the
510 major flavonoid from Melissa officinalis subsp officinalis. *Fitoterapia* **2000**, 71, 201-202.
511 34. Zaller, J. G.; Saccani, F.; Frank, T. Effects of earthworms and mycorrhizal fungi on the growth
512 of the medicinal herb Calendula officinalis (Asteraceae). *Plant Soil Environ*. **2011**, 57, 499-
513 504.
514 35. Hristozkova, M.; Geneva, M.; Stancheva, I.; Boychinova, M.; Djonova, E. Aspects of
515 mycorrhizal colonization in adaptation of sweet marjoram (Origanum majorana L.) grown
516 on industrially polluted soil. *Turkish. J. Biol.* **2015**, 39, 461-468.
517 36. Zubek, S.; Stefanowicz, A. M.; Blaszkowski, J.; Niklinska, M.; Seidler-Lozykowska, K.
518 Arbuscular mycorrhizal fungi and soil microbial communities under contrasting fertilization
519 of three medicinal plants. *Appl. Soil. Ecol*. **2012**, 59, 106-115.
520 37. Tabrizi, L.; Mohammadi, S.; Delshad, M.; Zadeh, B. M. Effect of arbuscular mycorrhizal fungi
521 on yield and phytoremediation performance of pot marigold (Calendula officinalis L.) under
522 heavy metals stress. *Int. J. Phytorem*. **2015**, 17, 1244-1252.
523 38. Gharib, F. A.; Moussa, L. A.; Massoud, O., N. Effect of compost and bio-fertilizers on growth,
524 yield and essential oil of sweet marjoram (Majorana hortensis) plant. *Int. J. Agric. Biol*.
525 **2008**, 10, 381-387.

526 39. Karagiannidis, N.; Panou-Filotheou, H.; Lazari, D.; Ipsilantis, I.; Karagiannidou, C. Essential oil
527 content and composition, nutrient and mycorrhizal status of some aromatic and medicinal
528 plants of northern Greece. *Nat. Prod. Commun*. **2010**, 5, 823-830.
529 40. Zubek, S.; Blaszkowski, J. Medicinal plants as hosts of arbuscular mycorrhizal fungi and dark
530 septate endophytes. *Phytochem. Rev*. **2009**, 8, 571-580.
531 41. Copetta, A.; Lingua, G.; Berta, G. Effects of three AM fungi on growth, distribution of
532 glandular hairs, and essential oil production in Ocimum basilicum L. var. Genovese.
533 *Mycorrhiza* **2006**, 16, 485-494.
534 42. De la Rosa-Mera, C. J.; Ferrera-Cerrato, R.; Alarcon, A.; Sanchez-Colin, M. D.; Munoz-Muniz,
535 O. D. Arbuscular mycorrhizal fungi and potassium bicarbonate enhance the foliar content
536 of the vinblastine alkaloid in Catharanthus roseus. *Plant Soil.* **2011**, 349, 367-376.
537 43. Nell, M.; Voetsch, M.; Vierheilig, H.; Steinkellner, S.; Zitterl-Eglseer, K.; Franz, C.; Novak, J.
538 Effect of phosphorus uptake on growth and secondary metabolites of garden sage (Salvia
539 officinalis L.). *J. Sci. Food Agric*. **2009**, 89, 1090-1096.
540 44. Lee, J.; Scagel, C. F. Chicoric acid found in basil (Ocimum basilicum L.) leaves. *Food Chem.*
541 **2009**, 115, 650-656.
542 45. Larose, G.; Chenevert, R.; Moutoglis, P.; Gagne, S.; Piche, Y.; Vierheilig, H. Flavonoid levels in
543 roots of Medicago sativa are modulated by the developmental stage of the symbiosis and
544 the root colonizing arbuscular mycorrhizal fungus. *J. Plant Physiol.* **2002**, 159, 1329-1339.
545 46. Jurkiewicz, A.; Ryszka, P.; Anielska, T.; Waligorski, P.; Bialonska, D.; Goralska, K.; Tsimilli-
546 Michael, M.; Turnau, K. Optimization of culture conditions of Arnica montana L.: effects of
547 mycorrhizal fungi and competing plants. *Mycorrhiza* **2010**, 20, 293-306.

548 47. Ponce, M. A.; Scervino, J. M.; Erra-Balsells, R.; Ocampo, J. A.; Godeas, A. M. Flavonoids from
549 shoots and roots of Trifolium repens (white clover) grown in presence or absence of the
550 arbuscular mycorrhizal fungus Glomus intraradices. *Phytochemistry* **2004**, 65, 1925-1930.
551 48. Palermo, M.; Colla, G.; Barbieri, G.; Fogliano, V. Polyphenol metabolite profile of artichoke is
552 modulated by agronomical practices and cooking method. *J. Agric. Food Chem*. **2013**, 61,
553 7960-7968.
554 49. Petersen, M.; Abdullah, Y.; Benner, J.; Eberle, D.; Gehlen, K.; Huecherig, S.; Janiak, V.; Kim, K.
555 H.; Sander, M.; Weitzel, C.; Wolters, S. Evolution of rosmarinic acid biosynthesis.
556 *Phytochemistry* **2009**, 70, 1663-1679.
557 50. Mandal, S. M.; Chakraborty, D.; Dey, S. Phenolic acids act as signaling molecules in plant-
558 microbe symbioses. *Plant Signal. Behav*. **2010**, 5, 359-368.
559

560 **Figure 1.** HPLC chromatograms (λ= 330 nm) of

561 **A)** control marjoram extract (peak numbers correspond to: 1. Apigenin-6,8-di-*C*-glucoside; 2.
562 Luteolin-7'-*O*-glucuronide; 3. Rosmarinic acid; 4. Apigenin-glucuronide; 5. Lithospermic acid A
563 isomer a; 6. Salvianolic acid B; 7. Apigenin);

564 **B)** control lemon balm extract (peaks numbers correspond to: 1. Caftaric acid; 2.Caffeic acid; 3.
565 Caftaric acid hexoside; 4.Rosmarinic acid; 5. Lithospermic acid A isomer a; 6. Lithospermic acid A
566 isomer b) and

567 **C)** control marigold extract (peak numbers correspond to: 1. Chlorogenic acid; 2. Quercetin-3-*O*-
568 rhamnosyl-rutinoside; 3. Isorhamnetin-3-*O*-rhamnosyl-rutinoside; 4. Rutin; 5. Isorhamnetin-3-*O*-
569 neohespedidoside (calendoflavoside); 6. Isorhamnetin-3-*O*-rutinoside (Narcissin); 7.
570 Dicaffeoylquinic acid; 8. Isorhamnetin-malonyl-glucoside)

571

572 **Figure 2.** Box plot graphs of content (A) and yield (B, multiplying the values of content by the biomass
573 dry weight) of rosmarinic acid in non-mycorrhizal (AMF-) and mycorrhizal (AMF+) marjoram plants

Table 1. UV-Vis spectra and mass spectral characteristics of phenolic compounds in marjoram

Peak no.	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	Main fragments MS^2 (m/z)	Identification
1.	12.4	270, 333	593.1519	473.1080	Apigenin-6,8-di-*C*-glucoside (vicenin-2)
2.	19.3	254, 266, 347	461.0727	285.039	Luteolin-7'-*O*-glucuronide*
3.	21.4	290sh, 328	359.0768	161.0239	Rosmarinic acid*
4.	22.0	267, 334	445.0776	269.0452	Apigenin-glucuronide
5.	23.9	290sh, 324	537.1019	493.1117, 359.0748, 135.0446	Lithospermic acid A isomer a
6.	24.5	255, 286, 308, 338sh	717.1456	519.0929, 321.0401	Salvianolic acid B
7.	30.1	288, 344	269.0425	117.0342	Apigenin*

** Confirmation of these compounds has been done by reference standards. All other peaks were tentatively identified compared with literature data [12, 31]. a: isomer; Rt: retention time; λ_{max}: maximal wavelength; [M-H]-: negative ion mode*

Table 2. UV-Vis spectra and mass spectral characteristics of phenolic compounds in lemon balm

Peak no.	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	Main fragments MS^2 (m/z)	Identification
1.	6.6	295sh, 327	311.0406	179.0339	Caftaric acid
2.	10.1	295sh, 322	179.0343	134.0307	Caffeic acid
3.	19.6	295sh, 327	473.0723	179.0349, 135.0450, 311.0419	Caftaric acid hexoside
4.	21.4	290sh, 328	359.0769	161.0244, 133.0296, 197.0456	Rosmarinic acid*
5.	23.8	290sh, 324	537.1030,	295.0612, 493.1139, 135.0451, 359.0770	Lithospermic acid A isomer a
6.	25.1	290sh, 328	537.1034	359.0764, 135.0443, 493.1136, 295.0601	Lithospermic acid A isomer b

* *Confirmation of these compounds has been done by reference standards. All other peaks were tentatively identified compared with literature data [13]. a, b: isomers; Rt: retention time;* λ_{max}*: maximal wavelength; [M-H]-: negative ion mode*

Table 3. UV-Vis spectra and mass spectral characteristics of phenolic compounds in marigold

Peak no.	Rt (min)	λ_{max} (nm)	Molecular ion $[M+H]^+$ (m/z)	Main fragments MS^2+ (m/z)	Molecular ion $[M-H]^-$ (m/z)	Main fragments MS^2 - (m/z)	Identification
1	8.2	295sh, 326	-	-	353.0874	191.0557	Chlorogenic acid*
2	14.9	255,355	757.2196	611.1615, 465.1035, 303.0503	755.2062	-	Quercetin-3-*O*-rhamnosyl-rutinoside
3	16.8	254, 355	771.2234	625.1759, 479.1183, 317.0653	769.2200	314.0422	Isorhamnetin-3-*O*-rhamnosyl-rutinoside
4	17.0	255,354	611.1596	465.1021, 303.0494	609.1452	300.0269	Rutin*
5	18.3	254,354	625.1761	479.1198, 317.0653	623.161	314.0428	Isorhamnetin-3-O-neohespedidoside (calendoflavoside)*
6	19.3	254,354	625.1768	479.1186, 317,0658	623.1623	315.0501	Isorhamnetin-3-*O*-rutinoside (Narcissin)*
7	20.7	296sh, 327	-	-	515.1189	353.0875, 191.0557	Dicaffeoylquinic acid
8	22.3	265sh,354	565.1181	317.0652	-	-	Isorhamnetin-malonyl-glucoside

** Confirmation of these compounds has been done by reference standards. All other peaks were tentatively identified compared with literature data [32]. Rt: retention time; λmax: maximal wavelength; [M-H]-: negative ion mode; [M+H]+: positive ion mode*

Table 4. Effect of inoculation on mycorrhizal status and yield parameters of marjoram, lemon balm and marigold

Plants	Treatments	Yield of herbs*						Root colonization		
		Fresh weight (g/pot)		Dry weight (g/pot)		Number of flowers (no./pot)		F(%)	M(%)	A(%)
Marjoram	AMF+	5[1].3 ± 10.4	a	17.8 ± 3.5	a	-		99.3 ± 1.4	82.2 ± 15.4	57.3 ± 20.9
	AMF-	33.2 ± 9.9	b	9.6 ± 2.8	b	-		nd.	nd.	nd.
Lemon balm	AMF+	127.9 ± 23.8	c	30.1 ± 4.7	c	-		92.3 ± 11.2	62.5 ± 11.6	15.7 ± 6.6
	AMF-	122.4 ± 28.2	c	29.7 ± 5.5	c	-		nd.	nd.	nd.
Marigold	AMF+	35.6 ± 5.5	d	4.8±0.7	d	70 ± 9.1	a	51.6 ± 21.7	17.1 ± 12.0	4.6 ± 5.9
	AMF-	35.7 ± 5.5	d	4.8±0.7	d	58 ± 8.4	b	nd.	nd.	nd.

**flowering shoots of marjoram, leafy shoots of lemon balm, flowers of marigold; F(%): mycorrhizal frequency, M(%): intensity of colonization, A(%) arbuscule richness; AMF+: mycorrhizal plants; AMF-: non-mycorrhizal plants; nd.: not detected; Means(n=12) followed by standard errors are presented. Different lower-case letters mark the significant differences between the coherent values (p<0.05, Mann-Whitney U Test).*

Table 5. Effect of AMF inoculation on polyphenol content and yield of marjoram

Treatment	Apigenin-6,8-di-C-glucoside	Luteolin-7'-O-glucuronide	Rosmarinic acid	Apigenin-glucuronide	Lithospermic acid A isomer
	content of polyphenols (mg/g DW)				
AMF+	1.8 ± 0.02 a	3.4 ± 0.23 a	21.3 ± 0.51 a	1.8 ± 0.03 a	17.0 ± 0.46 a
AMF-	2.6 ± 0.03 b	3.8 ± 0.37 a	27.2 ± 0.75 b	2.0 ± 0.03 a	21.0 ± 0.75 b
	yield of polyphenols (mg/pot)				
AMF+	31.2 ± 0.27 c	60.5 ± 4.06 b	379.2 ± 9.13 c	32.4 ± 0.49 b	303.2 ± 8.16 c
AMF-	24.6 ± 0.33 d	36.1 ± 3.53 c	261.0 ± 7.22 d	18.2 ± 0.29 c	201.5 ± 7.17 d

Means (n=6) followed by standard errors are presented. Different lower-case letters mark the significant differences between the coherent values in each column ($p<0.01$, Mann-Whitney U Test); AMF+: mycorrhizal plants; AMF-: non-mycorrhizal plants, DW: dry weight; Yield of polyphenols was calculated by multiplying the values of content by the biomass dry weight

Table 6. Effect of AMF inoculation on polyphenol content and yield of lemon balm

Treatment	Rosmarinic acid	Lithospermic acid A isomer a	Lithospermic acid A isomer b
	content of polyphenols (mg/g DW)		
AMF+	66.3 ± 2.27 c	9.4 ± 0.36 c	34.2 ± 1.55 c
AMF-	59.7 ± 0.51 d	8.0 ± 0.13 d	29.9 ± 0.71 d
	yield of polyphenols (mg/pot)		
AMF+	1996.2 ± 68.42 e	281.9 ± 10.92 e	1030.6 ± 46.57 e
AMF-	1774.5 ± 15.22 f	237.3 ± 3.88 f	888.4 ± 21.04 f

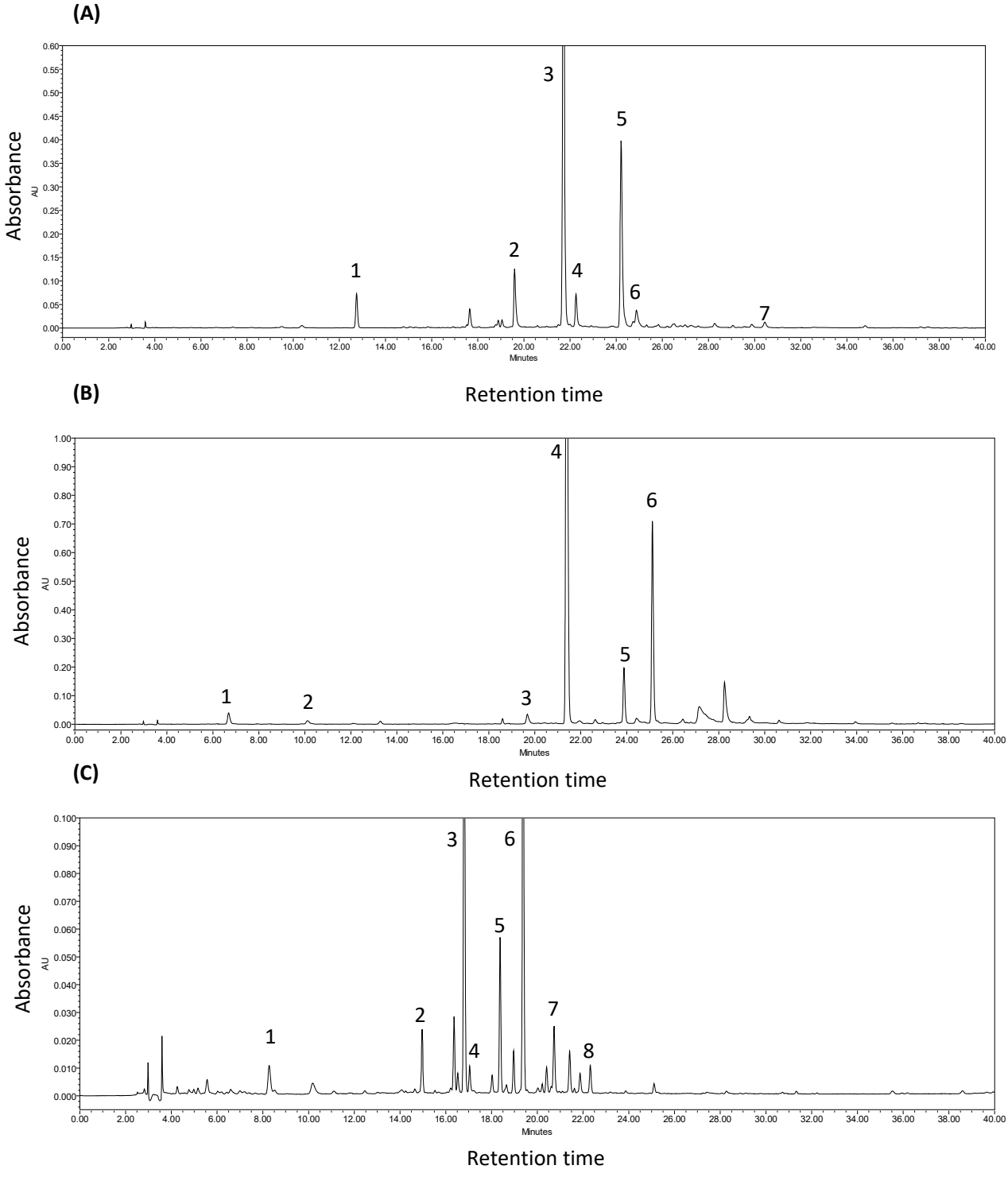
Means (n=6) followed by standard errors are presented. . Different lower-case letters (c, d, e, f) mark the significant differences between the coherent values in each column (p<0.01, Mann-Whitney U Test); AMF+: mycorrhizal plants; AMF-: non-mycorrhizal plants; DW: dry weight; a,b: isomers; Yield of polyphenols was calculated by multiplying the values of content by the biomass dry weight

Table 7. Effect of AMF inoculation on polyphenol content and yield of marigold

Treatment	Chlorogenic acid	Quercetin-3-*O*-rhamnosyl-rutinoside	Isorhamnetin-3-*O*-rhamnosyl-rutinoside	Rutin	Calendo-flavoside	Narcissin	Isorhamnetin-malonyl-glucoside
	content of polyphenols (mg/g DW)						
AMF+	0.3 ± 0.03 a	1.0 ± 0.05 a	8.0 ± 0.54 a	0.3 ± 0.02 a	1.9 ± 0.10 a	6.1 ± 0.55 a	0.5 ± 0.04 a
AMF-	0.3 ± 0.01 a	1.0 ± 0.01 a	8.5 ± 0.08 a	0.4 ± 0.01 a	1.8 ± 0.01 b	6.5 ± 0.11 a	0.4 ± 0,01 b
	yield of polyphenols (mg/pot)						
AMF+	1.5 ± 0.17 b	4.6 ± 0.22 b	38.3 ± 2.14 b	1.7 ± 0.11 b	9.0 ± 0.48 c	29.1 ± 2.62 b	2.3 ± 0.18 c
AMF-	1.4 ± 0.03 b	5.0 ± 0.05 b	40.8 ± 0.38 b	1.8 ± 0.03 b	8.6 ± 0.07 c	31.4 ± 0.54 b	1.8 ± 0.05 d

Means (n=6) followed by standard errors are presented. Different lower-case letters mark the significant differences between the coherent values in each column (p≤0.05, Mann-Whitney U Test); AMF+: mycorrhizal plants; AMF-: non-mycorrhizal plants; DW: dry weight; Yield of polyphenols was calculated by multiplying the values of content by the biomass dry weight

Figure 1.


(A)
Absorbance
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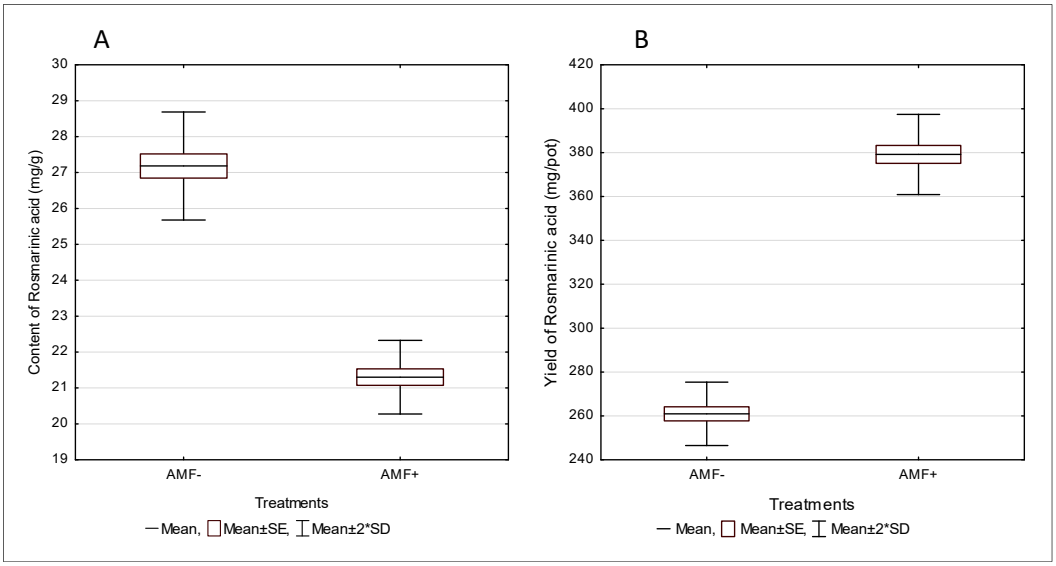
Retention time

Figure 2.


A
Content of Rosmarinic acid (mg/g)
30
29
28
27
26
25
24
23
22
21
20
19
AMF-
AMF+
Treatments
Mean, Mean±SE, Mean±2*SD
B
Yield of Rosmarinic acid (mg/pot)
420
400
380
360
340
320
300
280
260
240
AMF-
AMF+
Treatments
Mean, Mean±SE, Mean±2*SD