1 **Effect of Arbuscular Mycorrhizal Fungi on the Growth and the Polyphenol Profile of**

2 **Marjoram, Lemon Balm and Marigold**

3

4 RITA ENGEL[1*], KRISZTINA SZABÓ[1], LÁSZLÓ ABRANKÓ[2], KATA RENDES[3], ANNA FÜZY[4], TÜNDE

5 TAKÁCS[4]

6

7 1 MTA, Centre for Ecological Research, Institute of Ecology and Botany, Vácrátót, H-2163,

8 Alkotmány út 2-4, Hungary, engel.rita@okologia.mta.hu, 0036-28-360-122/251

9 2 Szent István University, Faculty of Food Science, Department of Applied Chemistry, Budapest,

10 H-1118, Villányi út 29–43, Hungary

11 3 Budapest University of Technology and Economics, Faculty of Chemical Technology and

12 Biotechnology, Budapest, H-1111, Műegyetem rkp. 3, Hungary

13 4 MTA Centre for Agricultural Research, Institute for Soil Sciences and Agricultural Chemistry,

14 Budapest, H-1022, Herman Ottó út 15, Hungary

1

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.

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

75 the arbuscular mycorrhiza (AM) is a mutualistic relationship between phytobionts and

76 mycobionts. AMF have direct and indirect effects on plant nutrient uptake, root morphology,

77 many physiological and developmental processes of plants [16]. These fungi are obligate

78 biotrophs, which must develop a symbiotic association with the host in order to grow and

79 complete their life cycle [17]. AM fungi take up products of host plant photosynthesis. Hexoses

80 transfer may occur through arbuscules as an intracellular interface of symbiotic association. In

81 contrast to the diverse array of terrestrial plants that form AM, only ca. 200-250

82 Glomeromycota species have so far been described, suggesting that mycobiont specificity is low

83 relative to their potential plant hosts [18]. The root colonization by AMF has been found to

84 increase the productivity of several crops due to a range of benefits provided to the host plants.

85 Positive effects of AM symbiosis on plant growth, water uptake, nutrition uptake and tolerance

86 to environmental stressors (drought, salt, heavy metal pollution, soil pathogens) have been

87 proved in numerous studies [19-21]. Apart from these beneficial effects, accumulation of

88 secondary metabolites such as essential oils, alkaloids and phenolic compounds in medicinal

89 plants can also be affected by this symbiosis inducing important changes in physiological

90 processes of host [19, 22, 23]. However, the way of this influence on the active substances is not

91 always evident. The presence of AMF might either enhance the accumulation of the secondary

92 metabolites in the plant's organs but in some cases there is no detectable effect of it. Recent

93 observations indicate that the influence of the AMF colonization on the level of secondary

94 metabolites highly depends on the type of host plant and the fungi species. For example, the

95 polyphenol content of *Ocimum basilicum* L. was increased as a result of the colonization by

96 *Glomus caledonium*, while the colonization by *Glomus intraradices* had no effect [24]. At the same

97   time, the inoculation with *Glomus intraradices* increased the polyphenol content of *Echinacea*

98   *purpurea* (L.) Moench. [25]. Consequently, the use of proper AMF during the cultivation can

99   improve the quantity as well as the quality of the obtained plant material.

100 Although the chemical composition and pharmacological activities of pot marigold, sweet

101 marjoram and lemon balm have been well studied and earlier findings show that these herbs

102 colonized by AMF, no data are available about the influence of AMF on the polyphenol content

103 of these important plant species. Thus, the aim of the present study is to examine the effect of

104 a commercially available AMF mixture on the profile and content of phenolic compounds of pot

105 marigold, sweet marjoram and lemon balm. Our findings allow us to evaluate the possibility of

106 eco-friendly agricultural application of AMF in order to improve the cultivation of selected

107 herbs.

108

109 **MATERIALS AND METHODS**

110

111 **Plant Material and Experimental Design.** Pot marigold, sweet marjoram and lemon balm were

112 chosen in this study since these species favorably form symbiotic relations with AMF, and they

113 are commercially important herbs. In addition, they are considered as excellent and diverse

114 model species since they represent two different plant life-forms (annual and perennial) and

115 moreover, different parts of them are utilized. Namely, flower of marigold, flowering shoots of

116 marjoram and leaves of lemon balm are used as drugs, i.e. the parts rich in active substances. A

117 pot experiment was performed in plant-growth room involving marigold, marjoram and lemon

118 balm grown with and without AM fungal inoculation. In order to ensure the maximal

6

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

141 shoots and leafy shoots were collected from each pot if there were any. All samples collected

142 from the same pot were pooled. For the analytical measurements pools were formed also from

143 the AMF-treated and non-treated samples. Fresh and dry weights of collected plant material

144 were weighed. Parts of the plants were dried at 40°C in drying oven. Diameters of the flowers

145 of marigold were measured, number of flowers per pot was counted. After drying, the sepals

146 were removed from the flowers of marigold, the stems were removed from the collected

147 shoots of marjoram and lemon balm. These purified plant materials were used for chemical

148 analyses.

149 **Root Colonization.** Root samples of control and mycorrhizal plants were taken at the middle

150 (3rd week, 3 replicates) and at the end of the vegetation period (15th week, 9 replicates). Root

151 samples of the three herbs were cleaned and stained by cotton blue (1 w/v%) solution [26, 27].

152 Mycorrhizal colonization was estimated by examining 1 cm-root segments (30 segments/root

153 sample) under dissecting microscope (40-60X; Olympus B71). AM fungal colonization was

154 expressed as mycorrhizal frequency (F%), intensity of colonization (M%) and arbuscule richness

155 (A%) [28].

156 **Chemicals and Reagents.** All reagents were of analytical grade. Acetonitrile, methanol (LC-MS

157 grade) and formic acid (~98%) were purchased from Merck (Merck, Darmstadt, Germany). A

158 Milli-Q ultrapure water system (Merck Millipore, Billerica, MA, USA) was used throughout the

159 study to obtain high purity water for the HPLC (high performance liquid chromatography)

160 analysis. Crystalline reference substances of luteolin 7-glucoronide, lithospermic acid A,

161 isorhamnetin 3-rutinoside, isorhamnetin 3-neohesperidose, rutin, apigenin, rosmarinic acid,

162 chlorogenic acid were purchased from Phytolab GmbH (Vestenbergsgreuth, Germany).

163   **Sample Preparation.** Sample preparation procedure was adopted from Abranko et al. [29] and

164   applied with slight modifications. An amount of 200 mg dried and ground plant material from

165   the pools of samples was weighed into a centrifuge tube (50mL), and 10 mL 60% aqueous

166   MeOH containing 1% formic acid was added. Samples were extracted for 1 hour in an ultrasonic

167   bath (<35 °C at the end). Extracts were centrifuged for 10 min at 8000 g. An aliquot of 2.5 mL

168   supernatant was transferred into a 15-mL graded centrifuge tube and diluted to 10 mL with

169   water. Finally, it was filtered (Cronus 25 mm PTFE Syringe Filter 0.22μm) before injecting to the

170   HPLC. In case of each sample, six replicates were extracted and analyzed separately.

171   **Instrumentation.** Identification of the major polyphenolic compounds was performed with a

172   high performance liquid chromatography (HPLC) system including a diode array detector (DAD)

173   coupled to an Agilent (Santa Clara, CA USA) 6530 quadrupole – time-of-flight mass

174   spectrometer (q-TOFMS), which was equipped with a dual spray ESI source. Quantitative

175   determination of the identified compounds was accomplished on a Waters Alliance HPLC-

176   system equipped with photodiode array detector (PDA) together with a quaternary pump, an

177   auto-sample injector, an on-line degasser and an automatic thermostatic column oven (Waters

178   Corp., Milford, MA, USA). The same chromatographic conditions (analytical column, mobile

179   phases, gradient program) were applied in both systems (described in detail below).

180   **HPLC-ESI-qTOFMS analysis.** The q-TOFMS was used with the following operation parameters:

181   capillary voltage, ±4,000 V; nebulizer pressure, 40 psig; drying gas flow rate, 13 l/min; gas

182   temperature, 350 °C. During these experiments, fragmentor voltage was triggered

183   automatically between 160 V and 210 V in positive mode and 140 and 240 V in negative mode.

184   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

207   substances were not available, calibration functions of similar available compounds were

208   employed to estimate their concentrations. Particularly, both lithospermic acid A isomers were

209   quantified based on the calibration function of lithospermic acid A, whereas apigenin-6,8-di-*C*-

210   glucoside and apigenin-glucuronide were quantified using the calibration function for apigenin

211   aglycone. Moreover, quercetin-3-*O*-rhamnosyl-rutinoside was quantified using the calibration

212   function of rutin, whereas the calibration function of isorhamnetin-3-*O*-rutinoside was used to

213   estimate the concentration of isorhamnetin-3-*O*-rhamnosyl-rutinoside and isorhamnetin-

214   malonyl-glucoside. A correction factor derived from the quotient of the molecular weight of the

215   compound used for calibration and that of the unknown one was applied in all cases to be able

216   to express results in mg/g dry weight [30].

217   **Statistical analysis.** Data of yield and polyphenol content were analyzed by non-parametric

218   Mann-Whitney U test ($p \leq 0.05$) using StatSoft's STATISTICA version 10.

219

220   **RESULTS**

221

222   **Identification of phenolic compounds. Figure 1. (a-c)** shows the phenolic compound profiles of

223   marjoram, lemon balm and marigold, respectively. Phenolic compounds were tentatively

224   identified based on UV spectra and accurate mass fragmentation information [12, 13, 29-32].

225   Retention time, UV-Vis and mass spectral characteristics, as well as tentative identification of

226   compounds are presented in **Table 1-3**. When standard substances were available, retention

227   time information was also used for compound confirmation. These compounds are labelled

228   with asterisk in **Table 1-3**. As shown in **Figure 1**. **(a-c)**, most compounds with prevalent UV

229   signal at 330 nm, a characteristic wavelengths of polyphenols having a subunit with a cinnamoyl

230   structure, were tentatively identified. In particular, seven phenolic compounds in marjoram, six

231   in lemon balm and eight in marigold. Among them the main compounds of marjoram and

232   lemon balm were rosmarinic acid and lithospermic acid A isomers, while in marigold

233   isorhamnetin-3-O-rhamnosyl-rutinoside and narcissin were the most abundant ones.

234   **AMF Root Colonization.** In the microbial treatment all three herb species formed symbiotic

235   relations with the added commercial AMF inoculum. No AMF colonization was detected in

236   control groups (**Table 4**.). The root system of the three plant species were colonized in different

237   rates. According to the root colonization parameters shown in **Table 4**., marjoram plants had

238   the highest level of fungal colonization followed by lemon balm and marigold. The AMF root

239   colonization in marjoram reached almost 100% after 15 weeks of growth, and the arbuscle

240   density (A%) was also very high. In lemon balm, the AMF root colonization can be characterized

241   by high intensity of colonization but low arbuscule richness. The intensity of colonization and

242   the arbuscule density in marigold were very low compared to the other two species (**Table 4**.).

243   **Yield.** Mycorrhization significantly increased the yield of flowering shoots in marjoram. The AM

244   plants had more than 1.5 times higher biomass production compared to non-mycorrhizal

245   plants. The AMF treatment did not cause significant differences in the yield of lemon balm

246   (leafy shoots) and marigold (flowers). However, a significant difference was observed in the

247   number of marigold's flowers. AMF inoculation significantly increased the number of flowers.

248   Thus AM plants had a higher number, nonetheless smaller flowers compared to the control

249   plants (**Table 4**.).

250 **Polyphenols.** Quantitative data of main phenolic compounds of marjoram, lemon balm and

251 marigold are summarized in **Table 5-6**. In terms of polyphenol content, the effect of

252 mycorrhization was different in the three examined herbs. In the case of marjoram, a significant

253 decrease of three active substances was observed in plants colonized by AMF (**Table 5**.). In

254 these plants, the two main phenolic compounds, namely rosmarinic acid and lithospermic acid

255 A isomer were presented in 28 and 24 % lower concentration than in the non-mycorrhizal

256 control plants. The highest level of decrease (50%) was detected in apigenin-6,8-di-*C*-glucoside

257 content. Regardless of the lower concentration of the above mentioned compounds,

258 mycorrhization increased the total yield of polyphenols calculating on the yield of biomass

259 (**Table 5**.). The total yield of polyphenols was calculated by multiplying concentration by the

260 biomass dry weight. **Figure 2.** demonstrates the alternation between the content and yield of

261 polyphenols through the example of rosmarinic acid. With regard to lemon balm, the

262 mycorrhization also influenced the concentration of phenolic acids, but partially in a different

263 manner. In contrast to marjoram, the content of the main phenolic compounds in AMF

264 inoculated lemon balm was 11-17 % higher than in control plants. Since the mycorrhization had

265 no significant effect on the yield of biomass, the total yield of polyphenols showed similar

266 tendency to the content of polyphenol (**Table 6**.). Regarding marigold, significant changes were

267 only observed in the concentration of calendoflavosid and isorhamnetin-malonyl-glucoside.

268 These compounds were present at 5-25 % higher concentrations in the flowers of AMF treated

269 plants as compared to the non-mycorrhizal ones. However, this significant difference appeared

270 only in isorhamnetin-malonyl-glucoside concerning the total yield (**Table 7**.)

271

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

14

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

16

338 colonized *by Glomus intraradices* and *Gigaspora rosea* than in roots colonized by *G. mosseae* [45].

339 In case of *Echinacea purpurea* L., colonization with *G. intraradices* significantly increased the

340 content of phenolics in the root, especially of cynarin and cichoric, caftaric and chlorogenic acid

341 [25]. Mycorrhization was also related to increased concentration of total phenolic acids in the

342 root of *Arnica montana* L. [46]. Furthermore, in the root of *Trifolim repens* inoculated with

343 *Glomus intraradices* quercetin, acacetin and rhamnetin were detected, which were not

344 presented in the root of non-inoculated plants [47].

345 To the best of our knowledge this is the first time when the effect of mycorrhization was

346 investigated on the polyphenol profile of the aerial parts of marjoram, lemon balm and

347 marigold. According to our results, inoculation with AMF mixture did not change the

348 composition of the main polyphenols, but concentrations of certain compounds were

349 influenced. It was shown that mycorrhizal symbiosis clearly increased the content and the total

350 yield of rosmarinic acid and lithospermic acid A isomers in lemon balm. Our results also showed

351 that mycorrhization not uniformly affects polyphenol content in the studied herbs. For

352 instance, marjoram plants colonized by AMF showed a significant decrease in the content of

353 rosmarinic acid, lithospermic acid isomer and apigenin-6,8-di-*C*-glucoside in comparison with

354 non-inoculated plants. It should be noted that the decrease of polyphenol concentrations in

355 combination with the improved plant growth resulted in an overall improved polyphenol yield

356 in marjoram. Although the AMF inoculation did not cause any changes in the concentration and

357 the yield of major polyphenols of marigold, it significantly increased the total yield of a minor

358 component, namely isorhamnetin-malonyl-glucoside.

359 There are contradictory data in the literature about how AM symbiosis influences the

360 polyphenol content in the aerial parts of medicinal plants. Hristozkova et al. [35] investigated the

361 effect of inoculation with two strains of *Claroideoglomus claroideum* and a *Glomus mosseae* on

362 the total phenol and flavonoid concentration in the aerial part of marjoram. They found a

363 significant decrease in total phenol concentration in plants colonized by *G. mosseae*. While the

364 total flavonoid concentration was higher in the plants inoculated by one of the *C. claroideum*

365 strains compare with the other treatments. While *G. mosseae* did not affect the total flavonoid

366 content of marjoram, enhancement of total flavonoid content was observed in the flowers of

367 marigold plants colonized by *Glomus mossea* and *G. intraradices* [37]. In terms of AM symbiosis,

368 various data can also be found related with *Ocimum basilicum* L. another *Lamiaceae* species in

369 which also rosmarinic acid is one of the dominant phenolic acids. In an investigation of

370 functional diversity among three *Glomus* species (*G. intraradices*, *G. caledonium*, *G. mosseae*)

371 *Glomus caledonium* increased the rosmarinic acid content in the shoots of basil, while the

372 others in this aspect remained ineffective [24]. In another study, similarly to the above

373 mentioned, the inoculation with *G. intraradices* had no significant effect on polyphenolic

374 content and profile of the leaves of basil, but increased the total anthocyanin concentration [44].

375 Enhancing effect of AMF-inoculation on the total phenolic content in the leaves of

376 *Catharanthus roseus* and modifying effect on the polyphenol profile of artichoke have been

377 reported [42, 48].

378 Our findings in accordance with literature data suggest that the mycorrhizal influence on the

379 secondary metabolite pathways is a result of a complex mechanism depending on several

380 circumstances such as the species of fungus and host plant, the rate of mycorrhization, nutrient

381 content of the soil, health condition of the plants, environmental factors, amongst others.

382 There are several theories for the reasons of changes in polyphenol content induced by

383 mycorrhization. One of the possible explanations for the increased phenolic acid concentration

384 is related to improved nitrogen uptake. Since tyrosine and phenylalanine are the main

385 precursors of rosmarinic acid, the higher nitrogen uptake in AM plants might have contributed

386 to the increased production of these amino acids [24, 49]. As a possible option, AMF induced

387 changes in phytohormone level in host plants could be in the background of this phenomenon

388 [24]. Another reason for the positive and negative alternation of phenolic acid content in AM

389 plants might be that the polyphenols can act as signaling and/or as regulatory compounds in

390 plant-microbe symbioses [45, 50]. Nevertheless further in-depth research is required in order to

391 have more information on the role of the AMF in the accumulation of active ingredients in

392 herbs and to understand the background mechanisms. The interaction of the host plants with

393 the AMF depends on the genotype of the partners and on several environmental factors. In

394 contrast to the large number of herbs that form AM, limited knowledge is still available about

395 their mycorrhizal dependence and AMF preference.

396 Overall, this was the first time when the effect of mycorrhization on the polyphenol profile of

397 marjoram, lemon balm and marigold was determined. Results of the present study show that

398 AMF can provide different services for each herb. For instance while marjoram benefits more

399 from the AMF colonization in terms of growth, lemon balm has higher content of phenolic

400 acids. It shows that the effect and outcome of mycorrhization depends on the plant and also

401 the fungal species. It was shown that the accumulation and alternation of different polyphenols

402 in herb as the result of mycorrhizal colonization is a species specific mechanism. The results

403 suggest that the application of optimized AM fungal inoculum in the cultivation of medicinal

404 plants can be a great potential to improve the quantity and quality of the raw material. Thus

405 AMF as an ecosystem service can play a key role in sustainable agriculture.

406 Our further aim is to set up an open field experiment in order to examine the effect of the AMF

407 inoculation in the agricultural practice. Selection of compatible symbiotic partners and

408 application of AM fungi adapted functionally to the site specific conditions can be a successful

409 innovation for the future of plant cultivation.

410

421

422   **REFERENCES**

423   1. Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and

424   effects on health. *Nat. Prod. Rep.* **2009**, 26, 1001-1043.

425   2. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and

426   bioavailability. *Am. J. Clin. Nutr.* **2004**, 79, 727-747.

427   3. Scalbert, A.; Manach, C.; Morand, C.; Remesy, C.; Jimenez, L. Dietary polyphenols and the

428   prevention of diseases. *Crit. Rev. Food. Sci. Nutr.* **2005**, 45, 287-306.

429   4. Okuda, T. Antioxidants in Herbs: Polyphenols. In Antioxidant Food Supplements in Human

430   Health, Packer, L.; Hiramatsu, M.; Yoshikawa, T., Eds. Academic Press: California, USA, 1999;

431   pp 393-410.

432   5. Mieseler, T. Calendula An Herb Society of America Guide. http://www.herbsociety.org

433   (22/01/2016).

434   6. Meyers, M. Oregano and Marjoram An Herb Society of America Guide to the Genus

435   Origanum. http://www.herbsociety.org (22/01/2016).

436   7. Meyers, M. Lemon Balm: An Herb Society of America Guide. http://www.herbsociety.org

437   (22/01/2016).

438   8. *WHO Monographs on Selected Medicinal Plants*. World Health Organization: Geneva, 1999;

439   Vol. 2.

440   9. *European Pharmacopoeia 6th* ed. Council of Europe: Strasbourg France, 2007; Vol. 1.

441   10. Lim, T. K. Calendula officinalis. In Edible Medicinal And Non-Medicinal Plants, Flowers, Lim,

442   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.

463   18. Heijden, M. G. A.; Scheublin, T. R.; Brader, A. Taxonomic and functional diversity in

464   arbuscular mycorrhizal fungi - is there any relationship? *New Phytologist* **2004**, 164, 201-

465   204.

466   19. Gianinazzi, S.; Gollotte, A.; Binet, M.-N.; van Tuinen, D.; Redecker, D.; Wipf, D. Agroecology:

467   the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* **2010**, 20, 519-

468   530.

469   20. Gosling, P.; Hodge, A.; Goodlass, G.; Bending, G. D. Arbuscular mycorrhizal fungi and organic

470   farming. *Agric. Ecosyst. Environ*. **2006**, 113, 17-35.

471   21. Takács, T. Site-specific optimization of arbuscular mycorrhizal fungi mediated

472   phytoremediation. In Toxicity of heavy metals to legumes and bioremediation 1ed.; Zaidi,

473   A.; Ahmad, W. P.; Khan, M. S., Eds. Springer-Verlag: Berlin Heidelberg, 2012; pp 179-202.

474   22. Zeng, Y.; Guo, L. P.; Chen, B. D.; Hao, Z. P.; Wang, J. Y.; Huang, L. Q.; Yang, G.; Cui, X. M.;

475   Yang, L.; Wu, Z. X.; Chen, M. L.; Zhang, Y. Arbuscular mycorrhizal symbiosis and active

476   ingredients of medicinal plants: current research status and prospectives. *Mycorrhiza* **2013**,

477   23, 253-265.

478   23. Chen, Y. L.; Li, J. X.; Guo, L. P.; He, X. H.; Huang, L. Q. Application of AM Fungi to Improve the

479   Value of Medicinal Plants. In Mycorrhizal Fungi: Use in Sustainable Agriculture and Land

480   Restoration, Solaiman, Z.; Abbott, L. K.; Varma, A., Eds. Springer-Verlag: Berlin Heidelberg,

481   Germany, 2014; pp 171-187.

482   24. Toussaint, J. P.; Smith, F. A.; Smith, S. E. Arbuscular mycorrhizal fungi can induce the

483   production of phytochemicals in sweet basil irrespective of phosphorus nutrition.

484   *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

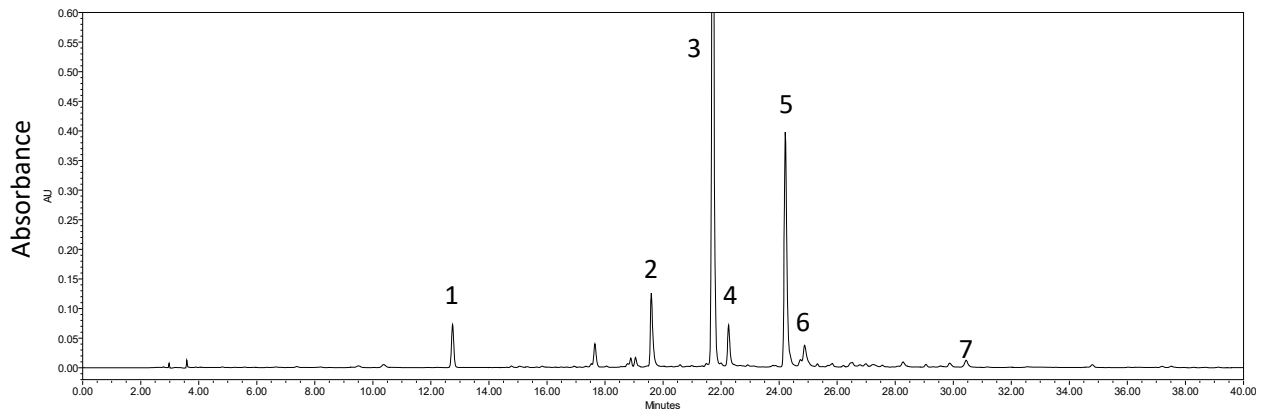*Means (n=6) followed by standard errors are presented. Different lower-case letters mark the significant differences between the coherent values in each column (p≤0.05, Mann-Whitney U Test); AMF+: mycorrhizal plants; AMF-: non-mycorrhizal plants; DW: dry weight; Yield of polyphenols was calculated by multiplying the values of content by the biomass dry weight*
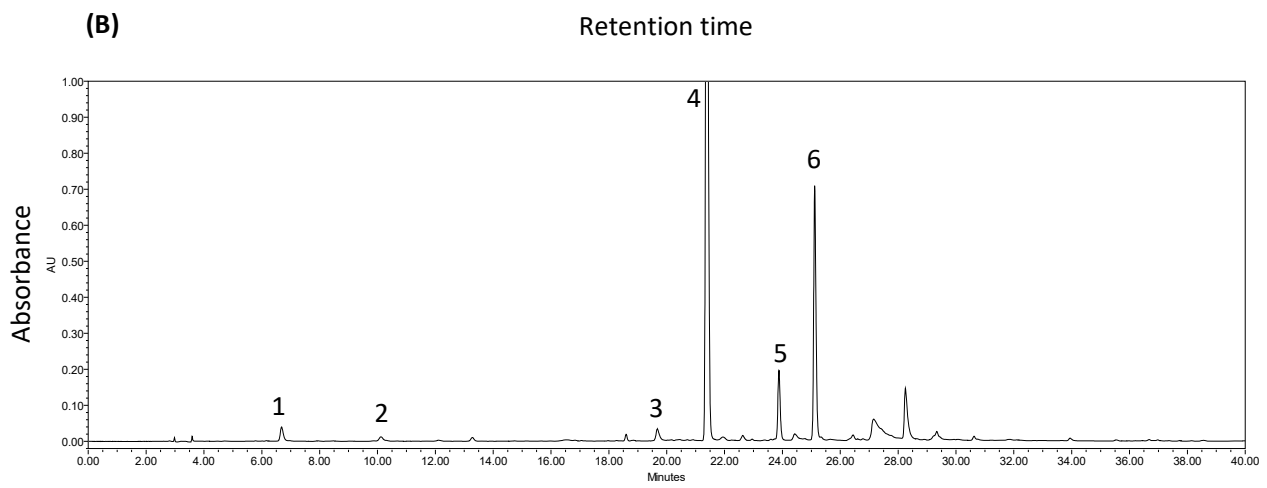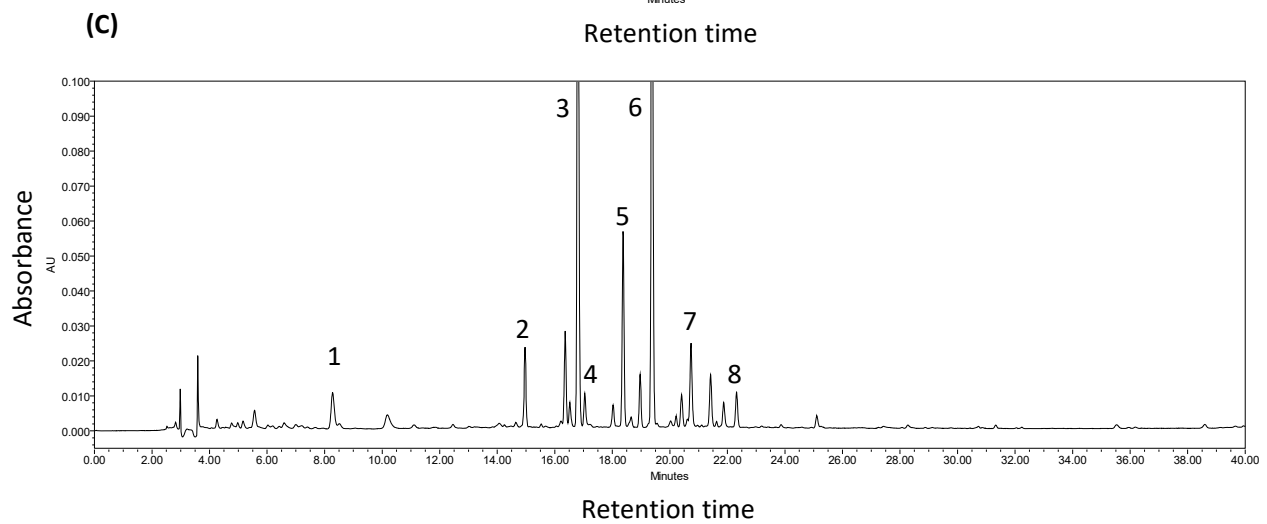
Figure 1.

(A)

(B)

(C)

Figure 2.