Corresponding Author: Dr. claudia ruta, Ph.D.

Corresponding Author's Institution: University

First Author: Waed Tarraf, Ph.D

Order of Authors: Waed Tarraf, Ph.D; claudia ruta, Ph.D.; Anna Tagarelli; Francesca De Cillis; Giuseppe De Mastro, Professor

Abstract: Arbuscular mycorrhiza fungi (AMF) contribute to the secondary metabolism and production of active ingredients in aromatic and medicinal plants. This symbiotic association is particularly affected by the availability of phosphorus (P) in the soil. This study was conducted on Salvia officinalis L. using two inocula, commercial Symbivit and Septoglomus viscosum (syn. Glomus viscosum), alone or supplemented with two doses of actual P (0.03, 0.06 g kg-1). The effects of these fungi and their combinations with P were determined in relation to the growth of sage plants (Regula variety), to the concentration of P in leaf tissues, and to the quantity and quality of essential oils (EOs). S. viscosum treated plants showed better growth with or without P-supply compared to non-mycorrhizal plants. The plants inoculated with S. viscosum presented the highest dry weight regardless of addition of P. Both AM fungi increased the leaf P content as more P was applied to the soil, whereas the EO content did not change with any of the treatments. Although the EO yield slightly increased with the Symbivit treatment, the chemical composition of the oil was drastically altered by S. viscosum in which the manool was the main component (28.13%), while α-thujone decreased (13.09%). These results suggest that AM symbiosis is a good candidate for promoting plant growth and essential oil composition and for improving P uptake in low fertility soils. Mycorrhizal technology can thus be considered as a sustainable strategy based on natural resources in order to influence the manool and α-thujone content in sage EO composition. These compositions are very important to develop new classes of biocides and contribute to reducing risks to both human health and the environment.

Cover Letter

Ms. Ref. No.: INDCRO-D-16-01910R1

Title: Influence of arbuscular mycorrhizae on plant growth, essential oil production and phosphorus uptake of Salvia officinalis L.

Industrial Crops and Products

Dear Editor (Mrs María Jesus Pascual Villalobos)

We provided, in a separate file, the responses of each reviewer comment.

Yours sincerely,

Claudia Ruta

Detailed Response to Reviewers

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Reviewers' comments:

Reviewer #2: I think that the kind of statistical elaboration proposed by me is
more suitable. Certainly, I know that one-way ANOVA is not totally wrong and
that the trial carried out by the authors is not a factorial trial. But I
suggested the two-factor ANOVA, because it could have given more reliable
statistical results.  However, to avoid any misunderstandings I'd propose to the
chief editor to send this article to another reviewer, perhaps more experienced
than me in statistical processing techniques, in order to have a third
evaluation.
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We are very sorry, but the design of our experiment does not permit us to follow your suggestion.

Our experimental design was not generated with two categorical factors including "AMP (Symbivit and S. viscosum)" and "(for example - AMP dosage/combination: Control, Symbivit/S. viscosum, Symbivit/S. viscosum + P1, Symbivit/S. viscosum + P2)" as suggested.

Treatment with AMF can be taken as a main factor, but we have great difficulties to follow up the suggestion to identify "AMP dosage / combination" as another factor because "AMP dosage / combination" itself is already an interaction.

Probably the two-factorial ANOVA proposed by you is due to a not completely clear description of the experimental design adopted in our manuscript.

More simply our experiment was generated as comparisons among the following 7 treatments:

1) Control (without mycorrhiza inoculation and mycorrhiza inoculation+P)

2) Symbivit

3) S. viscosum

4) Symbivit+P1 (0.03 g Kg^{-1} of substrate mixture)

5) Symbivit+P2 (0.06 g Kg^{-1} of substrate mixture)

6) S. viscosum+P1 (0.03 g Kg^{-1} of substrate mixture)

7) S. viscosum+P2 (0.06 g Kg^{-1} of substrate mixture)

According to this, we focused our investigation on comparing the effect of the AM fungi, their formulation with different doses of phosphorus and the control, as independent treatments of one factor. That is the reason why we performed one-way analysis of variance (ANOVA) of the data.

In order to clarify this misunderstanding, as mentioned above, we are submitting a revised version of the experimental design for which we performed the one-way analysis of variance (ANOVA) of the data.

In particular, we changed the text of the rows 122-131 with:

The pot experiment (three plants per pot) was generated as comparisons between the following 7

treatments, as independent treatments arranged in a complete randomized design with seven replicates:

1) Control (without mycorrhiza inoculation and mycorrhiza inoculation+P)

2) Symbivit

3) S. viscosum

4) Symbivit+P1 (0.03 g Kg^{-1} of substrate mixture)

5) Symbivit+P2 (0.06 g Kg^{-1} of substrate mixture)

6) S. viscosum+P1 (0.03 g Kg^{-1} of substrate mixture)

7) S. viscosum+P2 (0.06 g Kg^{-1} of substrate mixture);

and the text of the rows 205-208 with:

Comparisons among treatments were performed by one-way analysis of variance (ANOVA). The significant differences between treatments (indicated by different letters) were confirmed by Student-Newman-Keuls test at the 5% significance level. Statistical analyses were realized using Statistical Analysis System (SAS) 9.1.

We hope that solved the misunderstanding the revised version could be suitable for publication.

Highlights (for review)

- Mycorrhizal symbiosis is a very dynamic way to grow and develop sage plants according to P availability in the soil.
- *S. viscosum* led to an increase in biomass production compared to non-mycorrhizal plants, supporting the global trend to substitute the intensive application of chemical fertilizers with mycorrhizae.
- The essential oil composition strongly depended on mycorrhiza inoculums.
- *S. viscosum* maintained the concentration of α-thujone below ISO 9909 and produced a manool rich *S. officinalis* essential oil.
- Specific mycorrhizal strains help to achieve higher yields of the active compounds and/or improve the composition of the essential oil.

1   Influence of arbuscular mycorrhizae on plant growth, essential oil

2   production and phosphorus uptake of *Salvia officinalis* L.

3   Waed Tarraf, Claudia Ruta*, Anna Tagarelli, Francesca De Cillis, Giuseppe De Mastro

4   Department of Agricultural and Environmental Science, University of Bari, Bari- via Amendola, 165/A - 70126, Italy

5   *Corresponding author. E-mail: claudia.ruta@uniba.it

6

7   **Abstract**

8   Arbuscular mycorrhiza fungi (AMF) contribute to the secondary metabolism and

9   production of active ingredients in aromatic and medicinal plants. This symbiotic

10   association is particularly affected by the availability of phosphorus (P) in the soil. This

11   study was conducted on *Salvia officinalis* L. using two inocula, commercial Symbivit

12   and *Septoglomus viscosum* (syn. *Glomus viscosum*), alone or supplemented with two

13   doses of actual P (0.03, 0.06 g kg^{-1}). The effects of these fungi and their combinations

14   with P were determined in relation to the growth of sage plants (Regula variety), to the

15   concentration of P in leaf tissues, and to the quantity and quality of essential oils (EOs).

16   *S. viscosum* treated plants showed better growth with or without P-supply compared to

17   non-mycorrhizal plants. The plants inoculated with *S. viscosum* presented the highest

18   dry weight regardless of addition of P. Both AM fungi increased the leaf P content as

19   more P was applied to the soil, whereas the EO content did not change with any of the

20   treatments. Although the EO yield slightly increased with the Symbivit treatment, the

21   chemical composition of the oil was drastically altered by *S. viscosum* in which the

22   manool was the main component (28.13%), while α-thujone decreased (13.09%). These

23   results suggest that AM symbiosis is a good candidate for promoting plant growth and

essential oil composition and for improving P uptake in low fertility soils. Thus, mycorrhiza can be considered as a sustainable strategy based on natural resources in order to influence the manool and α-thujone content in sage EO composition. These compositions are very important to develop new classes of biocides and contribute to reducing risks to both human health and the environment.

Keywords: Biofertilizer; Mycorrhiza; Phosphorus; *Salvia officinalis*; Essential oil; Manool.

1. Introduction

Arbuscular mycorrhizal (AM) fungi are a fundamental group of soil microorganisms, classified in the phylum *Glomeromycota* (Schüßler and Walker, 2010), symbiotically associated with nearly 80% of plant species (Smith and Read, 2008). Their beneficial effects on the nutrition and development of plants has been clearly shown since the extra-radical mycelium surrounding the plant roots (Gosling et al., 2006), not only extends the volume of the soil (Azcón et al., 2003) but also makes the absorption of the nutritive minerals more efficient (Schnepf et al., 2011). Inoculation with AM fungi often facilitates the acquisition of poorly accessible nutrients in plants (Smith et al., 2003; Cavagnaro, 2008) particularly phosphate (P) (Smith et al., 2003) and thus promotes plant growth (Nell et al., 2010). Inoculation also facilitates the accumulation of more dry matter (Sharif and Claassen, 2011), improves the water uptake (Miransari, 2014) provides drought and salinity tolerance (Koide and Mosse, 2004; Campanelli et al., 2013), confers protection against pathogens (Filion et al., 1999) and influences the qualitative and quantitative profile of secondary metabolites (Zeng et al., 2013).

The lack of stable resources of medicinal and aromatic plants (MAPs) could be satisfied by the intensive application of chemical and organic fertilizers to optimize the productivity (Arabaci and Bayram, 2004) and increase the yield of oil (Khalid et al., 2006) and the main components (Ateia et al., 2009; Hendawy et al., 2010). In order to ensure the sustainability of agroecosystems (Barrios, 2007), many studies have also focused on increasing or enhancing the total yield and quality, using potential alternatives to substitute the existing inputs with renewable, less costly and more environmentally-friendly ones (Jeffries et al., 2003; López and Pascual-Villalobos, 2015). The biotechnological use of AM fungi in low-input cropping systems such as organic cultivation is of great importance to maintain or increase long-term soil fertility (Johansson et al., 2004) since their extensive mycelium can facilitate nutrients mobility in the soil (Bücking and Arjun Kafle, 2015). This suggest using of mycorrhiza as alternative to synthetic fertilizers that are not allowed in organic agriculture production.

Plant responses to the symbiotic establishment of arbuscular mycorrhizal (AM) fungus are mostly controlled by the availability of soil P, and are greatly limited by management practices in developed agriculture where P fertiliser is highly recommended (Smith and Smith, 2011). Expected AM benefits vary considerably depending on the P status of the soil; lower P levels increase root colonization (Zhu et al., 2005) whereas higher P levels inhibit root colonization (Smith and Read, 2008).

Studies on the formation of mycorrhizal symbioses between important species such as basil (*Ocimum basilicum* L.) (Toussaint et al., 2007), oregano (*Origanum vulgare* L.) (Khaosaad et al., 2006), mint (*Mentha piperita* L. (Cabello et al., 2005) and *Mentha arvensis* L. (Freitas et al., 2004)), sage (*Salvia officinalis* L.) (Nell et al., 2009) and AM fungi, have verified the function of AM in plant performance and nutrition. This has led

to variations in the yield or in the features of EOs, due to its great contribution in a range of chemical and biological parameters (Khaosaad et al., 2006), particularly the biosynthesis of terpenoids. Terpenoids are the principal components of essential oils require acetyl-CoA, ATP and NADPH, thus the synthesis of essential oil depends on the concentration of inorganic phosphorus in plants (Loomis and Corteau, 1972).

Salvia officinalis L. is the most widespread species of the Lamiaceae family (Avato et al., 2005), mainly found in Mediterranean areas (Menghini et al., 2013; De Mastro et al., 2006). Its biomass before flowering (European Pharmacopoeia, 2008) has been extensively used not only in food processing as a spice but also in pharmaceutical preparations showing a broad range of biological and medicinal activities (Menghini et al., 2013). These properties are related to the valuable ingredients consisting of terpenoids (camphor, 1,8-cineole and α-thujone and β-thujone) (Raal et al., 2007) and phenolics (flavone glycosides, caffeic acid and rosmarinic acid derivatives) (Dorman et al., 2003; Cuvelier et al., 1994).

The essential oil is the most important substance in the phytochemicals in sage. It is a volatile mixture, principally consisting of monoterpene, sesquiterpene and diterpene components (Fu et al., 2013) which are secreted and accumulated in specialized glandular trichomes of the plant (Schmiderer et al., 2010). With respect to the most prevalent constituents found in EOs, different chemotypes of sage oil have been identified (Mockutë et al., 2003) with versatile applications in the pharmacy and food sectors. The growth and developmental stages (Lakušić et al., 2013), environmental conditions (Arraiza et al., 2012), agricultural practices (Govahi et al., 2015), and plant organs (Verma et al., 2015) contribute mostly in the chemical composition and quality of the obtained oil.

94 Changing the concentration of EOs in medicinal plants as a result of AM establishment

95 has been attributed to a different nutritional status (Kapoor et al., 2002; Toussaint et al.,

96 2007), to an alteration in the phytohormonal balance (Allen et al., 1980, 1982) such as

97 the concentrations of auxins, cytokinins and gibberellins (Dixon et al., 1988; Torelli et

98 al., 2000), or to modifications in the structural tissues (glandular secreting trichomes)

99 (Malik et al., 2009). However, it is not clear how AM fungi cause changes in EOs, and

100 there are only a few studies on the growth and productivity of *S. officinalis* by which

101 different species of fungi promote the accumulation of secondary metabolisms in plant

102 tissues (Nell et al., 2009; Geneva et al., 2010).

103 The aim of this study was to extend the knowledge regarding screening the ideal strain

104 of AM fungi and the favourable soil P conditions for a successful symbiosis between

105 these medicinal and aromatic plants and AM fungi. Under greenhouse conditions the

106 role of mycorrhizal association was investigated in the cultivation of *S. officinalis* using

107 two AM fungal inoculums, *Septoglomus viscosum* and Symbivit, alone or combined

108 with different rates of P, in terms of plant growth, trichome density, biomass production

109 and both the quality and quantity of EOs.

110 **2. Materials and methods**

111 *2.1. Experimental setup*

112 The experiment used a soil collected from the experimental farm "Enrico Pantanelli" of

113 Bari University located in Policoro (southern Italy; 40°10′20″ N, 16°39′04″ E). This soil

114 was loamy, characterized by clay (Ø<2 µ) 22.82%, silt 37.40%, sand (2>Ø>0.02 mm)

115 39.78%, pH 8.32; 2.8% organic matter (Walkley-Black method), and 18.18 ppm

116 extractable P (Olsen method). Pots (20 cm in diameter) were filled with 3 kg of an

117 autoclaved (at 120 °C for 20 min) substrate mixture of sieved soil (pore size, 2 mm),

118   sand and perlite (1:1:1, v/v/v). Nutrients were mixed with the soil with the following

119   rates (mg dry soil kg^{-1}): K_2SO_4, 75; $CaCl_2 \cdot 2H_2O$, 75; $CuSO_4 \cdot 5H_2O$, 2.1; $ZnSO_4 \cdot 7H_2O$,

120   5.4; $MnSO_4 \cdot H_2O$, 10.5; $CoSO_4 \cdot 7H_2O$, 0.39; $MgSO_4 \cdot 7H_2O$, 45.0; $Na_2MoO_4 \cdot 2H_2O$, 0.18;

121   NH_4NO_3, 85.7, $Fe_6H_5O_7 \cdot 3H_2O$, 50.

122   The pot experiment (three plants per pot) was generated as comparisons between the

123   following 7 treatments, as independent treatments arranged in a complete randomized

124   design with seven replicates:

125   1) Control (without mycorrhiza inoculation and mycorrhiza inoculation+P)

126   2) Symbivit

127   3) *S. viscosum*

128   4) Symbivit+P_1 (0.03 g Kg^{-1} of substrate mixture)

129   5) Symbivit+P_2 (0.06 g Kg^{-1} of substrate mixture)

130   6) *S. viscosum*+P_1 (0.03 g Kg^{-1} of substrate mixture)

131   7) *S. viscosum*+P_2 (0.06 g Kg^{-1} of substrate mixture)

132   *2.2. Plant material and growth conditions*

133   Seeds of *Salvia officinalis* L., cv Regula (mediSeeds Sàrl) were sown in a sterilized

134   substrate. Plants were grown under a controlled glasshouse, with a temperature of 23-25

135   °C and relative humidity of 50% during the day and night. Two months after the

136   beginning of the experiment, a second application of the same rates of nutrients was

137   done by fertirrigation (see Section 2.1).

138   *2.3. Mycorrhizal inoculants*

139   Pure cultures of (AMF) *Septoglomus viscosum* (syn. *Glomus viscosum*) were multiplied

140   at our laboratory on onion (*Allium cepa* L.) plants selected as host crops due to their

141   high mycotrophy according to Dalpé and Monreal, (2004). This inoculum contained

142   sand, soil, spores, external mycelium, and infected root fragments, whereas Symbivit

143   purchased from Mybatec S.R.L. (Lanskroun, Czech Republic) consisted of six species

144   of *Glomus* fungi (*G. etunicatum*, *G. microaggregatum*, *G. intraradices*, *G. claroideum*,

145   *G. mosseae*, *G. geosporum*). For each pot, 300 spores of *S. viscosum* (30 g) or Symbivit

146   (3 g) were distributed immediately below the seeds at the time of planting.

147   *2.4. Determination of AM root colonization*

148   After a growth period of four months, the plants were harvested and divided into roots,

149   stems and leaves. Samples of roots were clarified and stained following Phillips and

150   Hayman (1970). The mycorrhizal colonization was estimated under an optical

151   microscope (Leica DMLB100, mark, Milan, Italy), considering the presence or absence

152   of fungal structures in the roots, in 1 cm segments by the following equation (Biermann

153   and Linderman, 1981):

$$\text{Percentage of root colonization} = \frac{\text{Total number of infected segments}}{\text{Total number of root segments examined}} \times 100$$

154   *2.5. Morphological measurements*

155   Number of leaves per plant was measured before placing them in a ventilated oven at 45

156   ºC until constant weight to measure the dry weight. Leaves from each treatment with the

157   same age and position on each plant, were randomly excised and examined by

158   stereomicroscopy (Leica DMLB100, mark, Milan, Italy). Moreover, glandular trichome

159   density was evaluated in the central portion of an abaxial surface of nine representative

7

160 leaves for each plant. The images were taken by a stereomicroscope connected to a PC

161 using the X-Pro analytical software (Alexasoft, Florence, Italy).

162 *2.6. P concentration in leaves*

163 Phosphorus was determined according to Hanson (1950). Approximately 1 g of dried

164 material was ground and incinerated at 550 °C for 1-2 days (white colour). Digestion

165 was carried out in 100 ml volumetric flasks containing 20 ml of hydrochloric acid HCl 1

166 N, with the help of a water bath for 30 minutes. The samples were filtrated using

167 filtration paper (Whatman n. 42), then diluted to 100 ml with distilled water. Two ml of

168 the filtered sample with 10 ml of ammonium molybdate, 2 ml of ascorbic acid solution

169 and about 8 ml of distilled water were heated using a bain-marie for 30 minutes. Finally,

170 each sample was made up to a volume of 100 ml. Absorbance was then read at 700 nm

171 using a spectrophotometer (Ultrospec 2100 *pro*). A standard curve was prepared using a

172 range of P concentrations from 0 to 1 mg l^{-1}. To fit the range of the standard curve the

173 samples were diluted.

174 *2.7. Essential oil analysis*

175 *2.7.1. EO isolation*

176 Air-dried leaves (20 g) of 4-month old plants were subjected to hydrodistillation

177 (European Pharmacopoeia, 2007) for 3 hours, using a modified Clevenger-type

178 apparatus. The essential oil obtained was dried over anhydrous sodium sulphate, and

179 after filtration it was stored in glass vials covered with aluminium foil to prevent light

180 exposure at 4 °C, for further analysis.

181 *2.7.2. Gas Chromatography/ Mass Spectrography analysis*

182 Chemical analyses were carried out by an Agilent 6890N gas chromatograph coupled to

183 an Agilent mass spectrometer 5973N (Agilent Technologies, Cernusco sul Naviglio,

184 MI, Italy) equipped with a data processor: Agilent enhanced Chemstation MSD

185 G1701DA D.03.00.611 version. Volatile components were separated on a capillary

186 column HP-5MS (5%- phenylmethypolysiloxane, 0.25 μm × 30 m × 0.25μm film

187 thicknesses). The temperature of the injector and transfer line were set to 250 and 300,

188 respectively. The column was heated to 60 ºC, then programmed to 240ºC at 3 ºC min^{-1},

189 then increased to 280 ºC at 8 ºC min^{-1} (held 5 minutes). The total run time was set at 65

190 minutes for each sample. The following conditions were adopted: split ratio 1:25, at

191 flow 1.1 ml min^{-1}, with Helium as carrier gas, and injection volume of 1μL of essential

192 oil diluted in dichloromethane (1:300). A mixture of aliphatic hydrocarbons (C8 - C30;

193 Sigma, IT-Milan) in n-hexane was directly injected into the GC under the above

194 temperature program, in order to calculate the temperature programmed retention

195 indices (RIs) of peaks in the chromatogram.

196 All the mass spectra were acquired using the electron-impact (EI) mode with an

197 ionization voltage of 70 eV (Tirillini et al., 2009).

198 *2.7.3. EO identification*

199 The volatile compounds were identified based on the retention index and mass spectra

200 obtained from Wiley (1994), NIST (1995) and Adams (2001) libraries, as well as the

201 literature data. The content of each component corresponded to percentage peak areas

202 without using correction factors (Marriot et al., 2001).

203 *2.8. Statistical analysis*

204 Comparisons among treatments were performed by one-way analysis of variance

205 (ANOVA). The significant differences among treatments (indicated by different letters)

206 were confirmed by Student-Newman-Keuls test at the 5% significance level. Statistical

207 analyses were realized using Statistical Analysis System (SAS) 9.1.

208 **3. Results and discussion**

209 *3.1. AM colonization*

210 By measuring the percentage colonization as the most common indicator for the activity

211 of AM fungi (Smith and Smith 2011), the results of both AM fungi indicated a full

212 colonization established in the inoculated plants after four months of growth. The

213 highest percentage of colonization (88%) was reached by Symbivit (Fig. 1). A high

214 percentage of mycorrhizal infection (83%) on *S. officinalis* was reported by

215 Karagiannidis et al. (2012) with *G. lamellosum,* while a previous study with Symbivit

216 and *G. mosseae* (Nell et al., 2009) showed a lower efficiency (32%) in the mycorrhizal
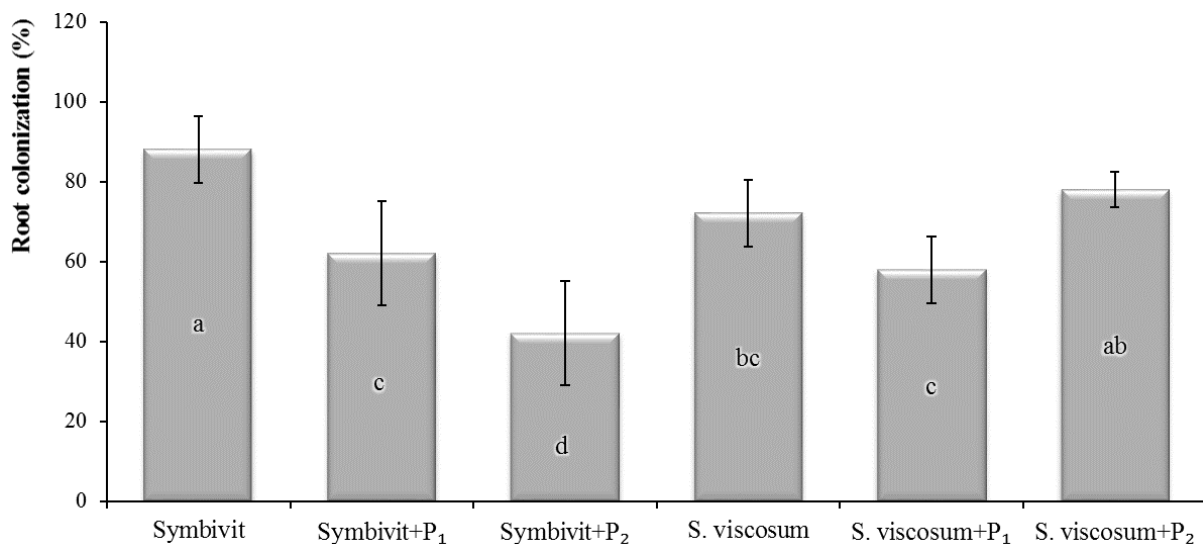
217 symbiosis with *S. officinalis.*

Fig. 1. Root colonization (percentages; mean±SD) of *Salvia officinalis* L. after treatment with P fertilization and mycorrhizal inoculation. Values with the same letters are not significantly different (P<0.05)

218   There was a significant effect of the P application on the AM inoculated plant regardless

219   of the fungus. In Symbivit inoculated plants, the P treatment, at the two doses, showed a

220   reduction in root colonization, while *S. viscosum* at the highest level (P_2) led to a slight

221   increase in the colonization percentage compared to *S. viscosum* inoculated plants (78%

222   *vs* 72%). This is in agreement with Karthikeyan et al. (2008) who recorded a maximum

223   level of root infection in the combined treatment of high P and *G. mosseae* on

224   *Catharanthus roseus*. In line with Zeng et al. (2013), these observations highlight the

225   variations in performance of the AM species and isolates used in this study.

226   Many studies have focused on the direct correlation between root colonization in plants

227   and P presence in soil. High levels of phosphorus usually lead to low AMF colonization

228   (Smith and Read, 2008; Duan et al., 2010). Kapoor et al., (2004) reported the same

229   result on mycorrhizal inoculation supplemented with P-fertilizer in *Foeniculum vulgare*

230   Mill. Also, Sanders (1975) revealed the contribution of P application in decreasing the

231   rate of mycorrhizal infection of onion roots. This might be attributed to a reduction in

232   intra and extra-radical AM development, thus suppressing the colonization (Abbott and

233   Robson, 1984; Liu et al., 2000).

234   The interaction between the target plant and fungal inoculation may also be significant

235   with high P soil fertility (Hamel et al., 1997; Vosátka, 1995), although its availability in

236   soil is a crucial factor for colonization occurrence (Smith and Read, 2008). The

237   tolerance of AM fungi to the nutrient supply has been explained by Ryan and Ash

238   (1999), as a result of lower initial soil P and N concentrations. In this context, the

239   amount of applied P did not substantially influence *S. viscosum* colonization, but it

240   affected Symbivit colonization. A notable decline was found when the plants were

241   inoculated with Symbivit under high P supply, which could be assigned to inhibition in

242   the development of spores and posterior root colonization (Urcoviche et al., 2015).

243   Thus, in terms of the effects of increasing P on AM fungi, the higher rate (264 mg kg^{-1})

244   was more compatible with the fungal *S. viscosum* inoculant and enhanced root

245   colonization (78%), while adding P reduced the percentage colonization in Symbivit to

246   42%.

247   According to their responses and adaptability to fertilization levels, AMF have been

248   grouped into insensitive and sensitive species (Bhadalung et al., 2005). The use of the *S.*

249   *viscosum* strain has equally been found to colonize sage roots at any given P dose and

250   therefore, it can be concluded that *S. viscosum* fungus is less sensitive to the P fertilizer

251   (Ryan and Ash, 1999). The existence of a high degree of root infection by *S. viscosum*

252   up to 58% in this study indicates that this fungus is more effective under adequate P

253   availability in the soil or in a nutrition schedule that includes phosphoric fertilization.

254   It is well known that the growth of AM is affected by the available levels of nutrients

255   (Abbott and Robson, 1984), above all P. From a practical point of view, sage

256   colonization is favored by a higher dose of P when the plants are inoculated with S.

257   *viscosum* and vice versa using Symbivit.

258   *3.2. Morphological measurements*

259   The number of leaves per plant was strongly affected by the symbiosis especially in

260   combination with P treatments (Table 1). In the AMF-host plants, *S. viscosum* (83.80)

261   was more effective than Symbivit (55.73), although the combination with the P

262   fertilization, in both, led to an increase in the number of leaves. The maximum number

263   of leaves per plant (97.83) was recorded in the plants inoculated with *S. viscosum* at a

264   lower level of P.

265 The effect of AM fungal inoculation influenced significantly the weight of leaves per

266 plant, although the weight of *S. viscosum* plants was higher (1.56 g d.w.) than Symbivit

267 (1.00 g d.w.) and the control plants (0.69 g d.w.) (Table 1). The presence of P increased

268 the dry weight in Symbivit inoculated plants.

Table 1
Effects of AM inoculants and AM+P fertilization dosage on number, dry weight, and P content of leaves

Treatments	Leaves (n.)	Leaf dry weight (g)	P in leaf tissues (mg g^{-1})
Control	29.78±2.17 d	0.69±0.07 b	1.70±0.02 c
Symbivit	55.73±5.92 c	1.00±0.20 b	2.08±0.33 c
Symbivit+P_1	89.89±4.76 ab	1.49±0.24 a	3.58±0.37 ab
Symbivit+P_2	85.89±4.48 ab	1.42±0.06 a	4.04±0.06 a
S. viscosum	83.80±8.87 b	1.56±0.19 a	2.04±0.06 c
S. viscosum+P_1	97.83±1.61 a	1.48±0.14 a	3.15±0.41 b
S. viscosum+P_2	94.17±3.62 ab	1.56±0.46 a	3.79±0.69 ab
Total mean	75.61	1.31	2.91
CV%	7.51	17.16	12.25

Values are means of seven replicates, values with the same letters are not significantly different (P<0.05)

269 The present results indicate the possibility to induce *S. officinalis* plants to have more

270 leaves than the control by using the AM inoculum, individually or together with P. In

271 agreement with the growth data of *Jatropha curcas* L. (Balota et al., 2011), an increase

272 in leaf number as a result of AMF inoculation or P-supply was observed, but did not

273 differ significantly in the basil plants inoculated with *G. fasciculatum* and *G. mosseae*

274 (Zolfaghari et al., 2013). The data obtained on the increase in the leaf number due to the

275 mycorrhizal symbiosis could be explained by an increase in the absorption surface area

276 of the roots provided by the action of extensive fungal hyphae on the plant growth (Liu

277 et al., 2007, Chaudhary et al., 2008).

278 The leaf biomass under mycorrhizal colonization increased regardless of inoculum 1.4-

279 2.3 times more than the uninoculated plants, while the highest weight was obtained by

280 *S. viscosum*+P_2 (Table 1). This was inconsistent with earlier published data (Nell et al.,

13

281   2009) where the leaf biomass of *S. officinalis* was significantly greater only in full and

282   half phosphorus treatments compared to inoculated treatments (*G. mosseae,* Symbivit

283   and *G. intraradices*).

284   The impact of fungal symbiosis has also been investigated in two genotypes of

285   *Origanum* sp. where the increase in leaf biomass correlated positively with mycorrhizal

286   infection (Khaosaad et al., 2006). The different species and isolates of AM fungi (Al-

287   Raddad, 1991; Zubek et al., 2010) could therefore be the main causes for the differences

288   in growth and development of mycorrhizal plants. Many species have presented a

289   considerable functional diversity in terms of the responsiveness to the same fungus

290   (Fernandez et al., 2009), and some varieties may also influence plant mycorrhizal

291   fungus interactions (Gupta et al., 2002). This diversity in AMF-host symbioses could be

292   influenced by the effectiveness of the fungus as symbiont and the responsiveness of

293   plants in terms of growth and P uptake (Smith et al., 2003). However, our experimental

294   data found a significant interaction between sage plants and mycorrhizae in terms of P

295   fertilization, which confirms the mutualistic relationship due to the functional activity of

296   the symbiosis in the exchange of carbon for phosphorus among the symbionts

297   (Helgason and Fitter, 2005). Plants provide the AM fungi with sugars, and fungi

298   enhance the accessibility of less mobile nutrients to plants (Smith and Smith, 1990).

299   *3.3. P concentration in leaves*

300   Phosphorus is often poorly available in the soil, due to insoluble calcium, iron and

301   aluminium phosphates or fixation to clay mineral surfaces (Smith and Read, 2008), and

302   is required by plants in large amounts. Thus, the soil used in the current study contained

303   18.18 ppm extractable P to prevent the plants from being P deficient. The data show that

304   the P concentration in leaf tissues from mycorrhizal plants (2.04 *vs* 2.08 mg g^{-1}) was not

14

305 significant compared to the non-mycorrhizal plants (1.70 mg g^{-1}) (Table 1). Nell et al.,

306 (2009) in a similar study revealed a positive effect of phosphorus leaf concentration in

307 sage due to the inoculation with *G. mosseae* and Symbivit. This is in contrast to the

308 observations by Copetta et al (2006) and Toussaint et al (2008) on *Ocimum basilicum*

309 who reported an absence of any favourable increase by either P addition or AM fungi on

310 the P levels in leaves and shoots.

311 In our experiment the addition of phosphorus to the AMF-inoculated plants resulted in a

312 significant increase in the P concentration in the leaves, with a tendency to be higher

313 with the maximum P-supply (Table 1). This phenomena, in the case of *S. viscosum*

314 inoculation, could confirm the efficiency of the AMF in minimizing the use of chemical

315 fertilizers considering that the application of P to the inoculated plants did not

316 significantly improve the leaf production (Table 1). Improving the absorption of P may

317 be attributed to the great capacity of AMF hyphae to explore more soil volume beyond

318 the depletion zone (Marschner and Dell 1994), and thus trigger P transport from the soil

319 to plant roots (George et al., 1992).

320 *3.4. Essential oil content and number of glands*

321 The essential oil concentration did not differ between treatments (Table 2). The mean

322 essential oil concentration for all treatments was 1.03% although the best results were

323 recorded in the Symbivit treatment (1.23%) (Table 2). Similarly, in the literature, the oil

324 content of sage oscillated around 1.5% (De Mastro et al., 2006; Menghini et al., 2013)

325 caused by different environmental conditions and harvest times. Earlier research on *S.*

326 *officinalis* by Nell et al., (2009) showed neither AM inoculation using Symbivit, *G.*

327 *mosseae* and *G. intraradices* or P application significantly changed the EO yields,

328 although other Lamiaceae species, *Mentha arvensis* (Freitas et al., 2004), *Ocimum*

329 *basilicum* (Copetta et al., 2006; Zolfaghari et al., 2013) and different *Origanum* species

330 (Khaosaad et al., 2006; Tarraf et al., 2015), demonstrated consistent results regarding

331 the increased oil quantity in favour of inoculation using AMF.

Table 2
Influence of AM inoculants and AM+P fertilization dosage on EO content and glands density

Treatments	Glands density (n/mm^2)	EO content (%)
Control	6.43±0.55 c	1.06±0.07
Symbivit	7.50±0.75 b	1.23±0.03
Symbivit+P$_1$	7.14±0.62 b	1.05±0.28
Symbivit+P$_2$	7.00±0.32 b	0.97±0.12
S. viscosum	9.32±1.22 a	0.88±0.17
S. viscosum+P$_1$	7.22±0.37 b	1.03±0.07
S. viscosum+P$_2$	7.59±0.44 b	0.99±0.20
Total mean	7.46	1.02
CV%	9.01	15.85

Values are means of seven replicates, values with the same letters are not significantly different (P<0.05)

332 Binet et al. (2011) have shown that AM did not affect the production of essential oils,

333 although the total biomass increased. Generally, the possible mechanisms in which AM

334 could alter the profile of secondary metabolism are correlated to the content of

335 cytokines or gibberellins (Copetta et al., 2006; Toussaint, 2007) or the density of

336 glandular trichome hairs in the case of EOs (Kapoor et al., 2007). AMF promote the

337 content of EOs in medicinal and aromatic plants; for example, Copetta et al., (2006)

338 indicated the role of three AM fungi in increasing the number of peltate trichomes and

339 consequently a higher synthesis of essential oils. The AM fungal symbiosis produced

340 more glands (Table 2) in line with the observations of Kappor et al. (2007) and

341 Zolfaghari et al. (2013). The number of glandular hairs was influenced by the P

342 fertilization dosage combined with AM inoculant (Table 2). In this study *S. viscosum*

343 exhibited the highest density of trichomes (9.32 glands/mm^2) over the control (6.43

344 glands/mm^2) followed by Symbivit and the combinations (7.00-7.59 glands/mm^2)

345 (Table 2). Similarly, Morone-Fortunato and Avato (2008) demonstrated an increase in

346 abundant oil-secretory glands in leaves of *Origanum vulgare* plants already inoculated

347 by *G. viscosum*. Although higher densities of glandular hairs were significantly present

348 on leaves of inoculated plants, the essential oil content was unaffected, since the

349 percentage did not show any differences between treatments.

350 *3.5. Essential oil composition*

351 In the essential oil from sage under all the treatments, the domination of oxygenated

352 monoterpenes was established (Table 3). This group is described as the major class of

353 EOs from sage, including α-thujone (cis-thujone), β-thujone (trans-thujone), camphor,

354 1,8-cineole, and borneol. We found a decreasing percentage in inoculated plants with *S.*

355 *viscosum* (31.90%) in this group, as against an almost constant percentage was found in

356 the other treatments (47.22-48.45%) (Table 3).

357 The oxygenated diterpenes were represented mainly by manool which showed a wide

358 range starting from the control leaves (13.57%) and increased by a factor of 2-fold with

359 *S. viscosum*. Manool increased from 1.2 to 1.6-times for most of the treatments, except

360 Symbivit indicated that the lowest increase was only 1.2-times above the control (Table

361 3). Oxygenated diterpenes were identified in all the essential oils. The results

362 demonstrated that EOs obtained from colonized plants alone or supplemented by P were

363 the richest in this component (Table 3). The highest amount (29.24%) was detected in

364 the essential oils obtained from *S. viscosum* inoculated plants in comparison to EOs

365 from the Symbivit treatment (16.71%). These outcomes highlight the significant

366 influence of different species of AM on the chemical profile of essential oils (Table 3).

367 In addition an increase in the EOs content was observed by *S. viscosum*, as well as

368 Symbivit under a range of P conditions (Table 3). The combined Symbivit essential oils

17

369 had more oxygenated diterpenes (20.30-20.88%) than Symbivit alone, while in *S.*

370 *viscosum* the opposite was found. Both *S. viscosum*+P_1 (22.54%) and *S. viscosum*+P_2,

371 (20.72%) revealed a lower content of this group than *S. viscosum* alone (Table 3). As a

372 result, the application of AM fungal colonization could benefit the production of EO

373 depending on the mycorrhizal and plant species.

374 AM fungi may influence the accumulation of mono- or diterpenes through the

375 enzymatic activity (Mandal et al., 2015) starting from their common precursor geranyl

376 diphosphate (GPP). Furthermore, the variability in terpenoid production in inoculated

377 plants may be due to the growth-promoting substances, induced by the microorganisms

378 (Zolfaghari et al., 2012), which may involve in the pathway of monoterpenes, such as

379 the cytokinin (El-keltawi and Croteau, 1987) or gibberellic acid (Prins et al., 2010). In

380 this context, the influence of AM fungi might suggest a possible explanation for plant

381 terpenoid variability since an enzymatic and phytohormone balance is up-regulated by

382 AMF symbiosis and consequently correlated to terpenoid levels.

383 The sesquiterpene hydrocarbons in the inoculated plants fertilized with the P were

384 significantly lower and closer (8.10-10.89%) to that of the control (10.03%) (Table 3).

385 On the other hand, the mycorrhizal EOs without an additional P application showed a

386 high percentage (13.80%, 13.69% for Symbivit and *S. viscosum*, respectively). The

387 minimal change was observed in oxygenated sesquiterpenes where their abundance

388 oscillated around ~15% regardless of the treatment. Only the *S. viscosum* essential oil

389 had a higher value (20.75%) than the control (12.35%) (Table 3). The relative content of

390 monoterpene hydrocarbons was 1.73-3.74%. The content in *S. viscosum* inoculated

391 plants was lower (1.73%) even with an increased P-supply (2.97-2.89%) than Symbivit

392 (3.10%) which increased the accumulation in plants fertilized with P (3.36-3.74%),

393 however it was less than non-colonized plants (4.16%) (Table 3).

394 Several potential mechanisms are related to the terpenoid accumulation by AMF have

395 been reported in the literature such as modifications in plant morphology (Kapoor et al.,

396 2007), P availability (Mandal et al., 2015) and gene transcription involved with

397 terpenoid biosynthetic pathways (Floß et al., 2008). Moreover, the dominance of one or

398 more compounds in the essential oil of sage should be attributed to the activation of the

399 particular metabolic pathway (Avato et al., 2005).

400 The essential oils under study contained about 44 compounds, representing ~99% of the

401 total area; these components with their retention index are listed in Table 3. The major

402 constituents recognized in sage oil were: α-thujone (13.09-28.34%), manool (13.57-

403 28.13%), camphor (8.37-13.51%), viridiflorol (9.41-16.94%), α-humulene (5.61-

404 8.49%), ß-thujone (2.22-5.58), 1.8-cineol (3.09- 5.09%), trans- caryophyllene (1.37-

405 4.48%), borneol (1.52-3.73%), α-humulene expoxide II (0.92-2.15%), and camphene

406 (0.55- 1.31%).

407 The most abundant compound was α-thujone whose content varied considerably (Table

408 3); lower values were found in AM-inoculated plants (13.09 and 21.61%) for *S.*

409 *viscosum* and Symbivit, respectively then increased in mycorrhizal plants with P

410 supplementation, irrespective of the AM inoculums: Symbivit+P_1 (26.40%),

411 Symbivit+P_2 (27.49%), *S. viscosum*+P_1 (26.19%), and *S. viscosum*+P_2 (25.05%).

412 The second most abundant compound was manool, which clearly increased with the

413 fungal symbiosis (Table 3). In line with this, the greatest proportion of manool was

414 found after mycorrhizal colonization using *S. viscosum* (28.13%) followed by *S.*

415 *viscosum*+P_1 inoculated treatment (22.32%) or the two combinations Symbivit with P_1

416   (19.98%) or P_2 (20.53%), as well as *S. viscosum*+P_2 (20.38%). Treating the inoculated

417   soil with P confirms the potential of each AM fungus to prompt an alteration in the EO

418   components. This may be related to the nutrition of the plants which, in turn, changes

419   the synthesis pathways, and the role of these compounds in plant physiology (Weisany

420   et al., 2015). The mycorrhizal colonization of root in the host plant has been found to

421   cause cytological changes of plastids and mitochondria, which results in the activation

422   of plastidial methylerythritol phosphate (MEP) pathway of isopentenyl pyrophosphate

423   (IPP) biosynthesis (Walter et al., 2000; Lohse et al., 2005). In this way, AM fungi can

424   induce the accumulation of terpenoides through increase of available P (Pedone-Bonfim

425   et al., 2015).

426   Mycorrhizal colonization as single treatment increased the amount of viridiflorol over

427   the control to 38% in the EOs from the Symbivit treatment and to 80% for the EOs from

428   *S. viscosum*. All the tested treatments generally had a positive effect on the viridiflorol

429   accumulation compared to the control (9.41%). In specific mycorrhizal plants the

430   treatments showed relatively high levels for the inoculation with *S. viscosum* and

431   Symbivit (16.94% and 13.02%, respectively) (Table 3). Adding phosphorus (P_1, P_2)

432   along with AM fungi decreased the viridiflorol content to 10.74% in Symbivit+P_1,

433   while it fluctuated at ~11% in the other treatments (Table 3). The quantity of α-

434   humulene mostly increased in all mycorrhizal plants both by *S. viscosum* (8.49%) and

435   by Symbivit (7.94%). An increase was also exhibited with low or high soil P with *S.*

436   *viscosum* (Table 3). Conversely, Symbivit along with the P fertilizer, decreased α-

437   humulene to 5.63%. The combined treatments produced more ß-thujone and 1.8-cineol

438   than mycorrhizal plants but less than non-mycorrhizal plants, except for Symbivit where

20

439   a single application led to 4.99% of 1.8-cineol compared to the other two combinations,

440   which showed lower percentages.

441   It was clearly evident that the abundance of both compounds was reduced by the

442   symbiotic association, as well as the different AM isolates. Therefore, the lowest values

443   (2.22, 3.09%) were detected in the essential oil derived from *S. viscosum* leaves, while

444   the other inoculum Symbivit recorded 3.18% and 4.99%, corresponding to ß-thujone

445   and 1.8-cineol, respectively (Table 3). In the present study, the sage essential oils from

446   the inoculated plants contained the lowest amounts of α-ß-thujone (15.31-24.78%), and

447   levels of camphor less than 12% (11.88, 8.37%), obtained by Symbivit and *S. viscosum*,

448   respectively (Table 3).

Table 3 Chemical components and compound groups of essential oils of *S. officinalis* after mycorrhiza and mycorrhiza+P treatments

N.	Component	RI[a]	R.T.	Control	Symbivit	Symbivit+P$_1$	Symbivit+P$_2$	*S. viscosum*	*S. viscosum*+P$_1$	*S. viscosum*+P$_2$
Monoterpenes hydrocarbons				**4.08**	**3.10**	**3.74**	**3.36**	**1.73**	**2.97**	**2.89**
1	α-pinene	**937**	5.18	0.78	0.60	1.01	0.81	Nd	0.74	0.67
2	Camphene	**953**	5.56	1.31	0.97	1.05	0.83	0.55	0.69	0.71
3	β-myrcene	**992**	6.65	0.40	0.27	0.29	0.31	0.19	0.28	0.27
4	α-terpinene	**1019**	7.46	0.19	0.13	0.15	0.14	0.10	0.13	0.11
5	ρ-cymene	**1027**	7.70	0.35	0.18	0.36	0.38	0.18	0.35	0.31
6	Limonene	**1032**	7.84	0.59	0.45	0.46	0.41	0.27	0.40	0.41
7	γ-terpinene	**1062**	8.86	0.27	0.21	0.20	0.22	0.16	0.19	0.19
8	α-terpinolene	**1090**	9.91	0.18	0.14	0.11	0.13	0.10	0.10	0.10
9	n-heneicosane	**2100**	47.39	Tr	0.15	0.11	0.13	0.19	0.10	0.11
Oxygenated monoterpenes				**57.32**	**48.45**	**48.90**	**49.30**	**31.90**	**47.22**	**48.03**
10	1-octen-3-ol	**980**	6.29	0.92	0.56	0.88	0.92	0.24	0.81	0.78
11	1,8-cineole	**1034**	7.93	5.09	4.99	3.52	3.34	3.09	4.39	4.78
12	Linalool	**1101**	10.38	0.11	0.14	Tr	Tr	Nd	Tr	Tr
13	α-thujone (cis-thujone)	**1107**	10.61	28.34	21.61	26.40	27.49	13.09	26.19	25.05
14	β-thujone (trans-thujone)	**1119**	10.99	5.58	3.18	5.00	4.99	2.22	4.46	4.50
15	Camphor	**1147**	12.06	13.51	11.88	10.24	9.40	8.37	8.82	10.13
16	Borneol	**1168**	12.94	2.27	3.73	1.74	1.95	3.09	1.52	1.80
17	Terpinen-4-ol	**1179**	13.41	0.25	0.28	0.27	0.30	0.25	0.26	0.21
18	α-terpineol	**1192**	13.96	0.11	0.14	0.12	0.11	0.12	0.12	0.10
19	Cis-dihydrocarvone	**1198**	14.27	0.13	Nd	0.10	Tr	Nd	Nd	Nd
20	Bornyl acetate	**1287**	17.92	0.86	1.80	0.63	0.68	1.28	0.56	0.67
21	Trans-sabinyl acetate	**1293**	18.23	0.15	0.15	Nd	0.10	0.15	0.10	Nd
Phenolic monoterpenes				**0.93**	**1.24**	**2.77**	**1.25**	**0.99**	**0.88**	**1.16**
22	Thymol	**1295**	18.30	0.53	0.57	2.34	0.90	0.45	0.51	0.75
23	Carvacrol	**1304**	18.68	0.41	0.68	0.43	0.36	0.54	0.37	0.42
Sesquiterpene hydrocarbons				**10.03**	**13.80**	**8.10**	**8.52**	**13.69**	**9.06**	**10.89**
24	Trans-caryophyllene	**1419**	23.40	2.84	4.48	1.37	1.73	2.89	1.56	2.41

22

	RI[a]								
25	α-humulene	**1454**	24.78	6.33	7.94	5.63	5.61	8.49	6.43	7.20
26	allo-aromadendrene	**1461**	25.06	0.12	0.14	0.11	0.11	0.14	0.11	0.11
27	α-selinene	**1494**	26.46	Tr	0.11	Tr	0.11	0.17	Tr	0.10
28	ð-cadinene	**1524**	27.61	Tr	Tr	Tr	Nd	0.12	Nd	Nd
29	α-trans-Bergamotyl acetate	**1804**	37.91	0.18	0.35	0.10	0.13	0.49	Tr	0.17
30	Longifolen	**2083**	46.87	0.57	0.78	0.88	0.83	1.39	0.97	0.90
Oxygenated sesquiterpene				**12.35**	**15.23**	**14.20**	**15.11**	**20.75**	**15.41**	**14.87**
31	Caryophyllene oxide	**1582**	29.83	0.31	0.28	0.21	0.29	0.33	0.30	0.30
32	Viridiflorol	**1591**	30.19	9.41	13.02	10.74	11.35	16.94	11.09	11.48
33	Carotol	**1597**	30.45	0.46	0.44	0.51	0.55	0.62	0.62	0.41
34	Humulene epoxide I	**1601**	30.60	0.11	0.18	0.12	0.13	0.20	0.23	0.10
35	α-Humulene epoxide II	**1607**	30.83	1.55	0.92	1.74	1.80	1.54	2.15	1.78
36	Humulene epoxide III	**1628**	31.57	0.15	0.13	0.17	0.17	0.20	0.24	0.14
37	Isospathulenol	**1633**	31.78	0.36	0.26	0.72	0.68	0.75	0.65	0.65
38	(E)-14-hydroxy-9-epi-caryophyllene	**1660**	32.76	Tr	Nd	Nd	0.16	0.18	0.12	Nd
Diterpene hydrocarbons				**0.09**	**0.15**	**0.15**	**0.15**	**0.25**	**0.14**	**0.15**
39	Rimuene (Rosa-5,15-diene)	**1927**	41.96	Tr	0.15	0.15	0.15	0.25	0.14	0.15
Oxygenated diterpene				**13.80**	**16.71**	**20.30**	**20.88**	**29.24**	**22.54**	**20.72**
40	Sclareoloxide	**1906**	41.30	0.12	0.19	0.18	0.20	0.30	0.22	0.18
41	manool	**2053**	45.92	13.57	16.40	19.98	20.53	28.13	22.32	20.38
42	Sclareol	**2225**	51.02	0.11	0.12	0.14	0.16	0.81	Nd	0.17
Notidentified compounds				**0.57**	**0.51**	**0.64**	**0.60**	**0.62**	**0.79**	**0.66**
43	Unknown	**1869**	40.02	0.40	0.24	0.64	0.60	0.41	0.79	0.66
44	Unknown	**1780**	37.05	0.18	0.27	Nd	Tr	0.21	Nd	Tr
Total (%)				**99.17**	**99.20**	**98.80**	**99.18**	**99.17**	**99.01**	**99.38**

[a] RI: Retention index on a HP-5 MS column; [b] Nd: Not detected; Tr: Traces <0.1%

449   The ISO (1997) 9909 standards have defined the limits for some constitutes in sage EO.

450   The amount of the first major component, α-thujone, varied between 28.34 and 13.09%

451   and that of its isomer (β-thujone) from 5.58 to 2.22% in all the essential oils studied.

452   The above amounts are in agreement with ISO (1997) (18.0-43.0, 3.0-8.5 % for α-

453   thujone and β-thujone, respectively). Only *S. viscosum* significantly reduced the level of

454   α-thujone to 13.09% and thereby did not fulfil the requirement of ISO 9909 for

455   medicinal uses. From an industrial point of view, this oil with the lowest content of α-

456   thujone is of great interest in the alimentary sector since α-thujone is still not safe for

457   use as a food additive, because of its potential risk for consumer's health.

458   Our research suggests that mycorrhizal symbiosis could be used as a biotechnological

459   approach to control the toxic effects of α-thujone in food and medicine products. All the

460   combined treatments of *S. viscosum* fungus were close to the minimum value, whereas

461   the individual application did not fall within the range permitted. This was also

462   achieved via the Symbivit+P combinations revealing the potential of different species of

463   AM fungi to alter the chemical components in the oil, with the knowledge that Symbivit

464   alone almost accumulated an amount close to the limit (18.0%) of ISO (1997) 9909

465   standards. The lowest value of β-thujone was obtained from *S. viscosum* and it was the

466   only treatment that was found to be out of the range. No α-pinene was detected in *S.*

467   *viscosum* and no significant difference was shown among the other treatments. In all

468   samples, bornyl acetate was present in the lowest range between 0.56 to 1.80%

469   compared to the limits (≤2.5%), where the quantities remained constant and seemed

470   unaffected by the combinations. Of note was the highly significant variation obtained in

471   the mycorrhizal treatments (Table 3).

472 Manool was the second highest compound found in the essential oil extracted from *S.*
473 *viscosum*- mycorrhizal plants, in accordance with previously investigated corresponding
474 oils in different countries (Tucker and Maciarello, 1990; Mockutë et al., 2003;
475 Lawrence, 2003; Maksimović et al., 2007). This component has shown biocidal activity
476 against diverse microorganisms (Ulubelen et al., 1994, Topçu and Gören, 2007; Ugur et
477 al., 2010; Souza et al., 2011; Moreira et al., 2013), thus more production of manool
478 through mycorrhizal symbioses would be of a great value for future applications.

479 The changes in the chemical composition of essential oils were likely achieved as a
480 defensive response to fungal colonization/infection (Zubek et al., 2012) or via better
481 nutrition (Karagiannidis et al., 2011). This is in contrast with Khaosaad et al. (2006)
482 who concluded that an improvement in P acquisition did not increase the quantitative
483 profile of inoculated oregano oil but depended on the mycorrhiza-oregano association.
484 The results of this study revealed a high variation among the EOs obtained from sage
485 treated plants with regard to the abundance of bioactive compounds. This may derive
486 from the variability of associated AM fungi or could be ascribed to the effects of the
487 nutrition conditions (P) which was the major focus of the present study. The significant
488 effect of the synergistic relationship on sage oil characteristics may highly depend on
489 the AM isolates used in the experiment and their compatibility with the plant cultivar or
490 genotype (Gupta et al., 2002).

491 **4. Conclusions**

492 AM fungi could be exploited in the sustainable production of aromatic and medicinal
493 plants. Our results demonstrate that mycorrhizal symbiosis is a very dynamic way to
494 grow and develop sage plants according to P availability in the soil. The association
495 with *S. viscosum* led to an increase in biomass production compared to non-mycorrhizal

496   plants, thus the global trend to substitute the intensive application of chemical fertilizers

497   with mycorrhizae as an efficient biofertilizer is supported by this study.

498   We found that the potential use of AM-fungi to improve the quality of *S. officinalis*

499   essential oil meets ISO 9909 for medicinal purposes, and in particular the inoculation

500   with *S. viscoscum*. This inoculation maintained the concentration of α-thujone, which is

501   known to be more toxic than β-thujone, below ISO 9909 and produced a manool-rich *S.*

502   *officinalis* essential oil. This oil is an effective antimicrobial agent against bacteria, and

503   is already being exploited the biocides market. The effectiveness of the colonization in

504   increasing the percentage of manool was based on the inocula, thus specific strains

505   could help to the desired positive traits, achieve higher yields of the active compounds,

506   and/or improve the composition of the essential oil. Thus, more attention is required in

507   research into medicinal and aromatic plants and mycorrhizae, in order to improve the

508   quantity and quality of the standard *S. officinalis* essential oil (ISO 9909:1997) and to

509   differentiate its compositional profile. We believe this will contribute to developing new

510   classes of biocides and contribute to reducing risks to both human health and the

511   environment.

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Table 1

Effects of AM inoculants and AM+P fertilization dosage on number, dry weight, and P content of leaves

Treatments	Leaves (n.)	Leaf dry weight (g)	P in leaf tissues (mg g^{-1})
Control	29.78±2.17 d	0.69±0.07 b	1.70±0.02 c
Symbivit	55.73±5.92 c	1.00±0.20 b	2.08±0.33 c
Symbivit+P$_1$	89.89±4.76 ab	1.49±0.24 a	3.58±0.37 ab
Symbivit+P$_2$	85.89±4.48 ab	1.42±0.06 a	4.04±0.06 a
S. viscosum	83.80±8.87 b	1.56±0.19 a	2.04±0.06 c
S. viscosum+P$_1$	97.83±1.61 a	1.48±0.14 a	3.15±0.41 b
S. viscosum+P$_2$	94.17±3.62 ab	1.56±0.46 a	3.79±0.69 ab
Total mean	75.61	1.31	2.91
CV%	7.51	17.16	12.25

Values are means of seven replicates, values with the same letters are not significantly different (P<0.05)

Table 2

Influence of AM inoculants and AM+P fertilization dosage on EO content and glands density

Treatments	Glands density (n/mm^2)	EO content (%)
Control	6.43±0.55 c	1.06±0.07
Symbivit	7.50±0.75 b	1.23±0.03
Symbivit+P$_1$	7.14±0.62 b	1.05±0.28
Symbivit+P$_2$	7.00±0.32 b	0.97±0.12
S. viscosum	9.32±1.22 a	0.88±0.17
S. viscosum+P$_1$	7.22±0.37 b	1.03±0.07
S. viscosum+P$_2$	7.59±0.44 b	0.99±0.20
Total mean	7.46	1.02
CV%	9.01	15.85

Values are means of seven replicates, values with the same letters are not significantly different (P<0.05)

Table 3 Chemical components and compound groups of essential oils of *S. officinalis* after mycorrhiza and mycorrhiza+P treatments

N.	Component	RI[a]	R.T.	Control	Symbivit	Symbivit+P$_1$	Symbivit+P$_2$	*S. viscosum*	*S. viscosum*+P$_1$	*S. viscosum*+P$_2$
Monoterpenes hydrocarbons				**4.08**	**3.10**	**3.74**	**3.36**	**1.73**	**2.97**	**2.89**
1	α-pinene	**937**	5.18	0.78	0.60	1.01	0.81	Nd	0.74	0.67
2	Camphene	**953**	5.56	1.31	0.97	1.05	0.83	0.55	0.69	0.71
3	β-myrcene	**992**	6.65	0.40	0.27	0.29	0.31	0.19	0.28	0.27
4	α-terpinene	**1019**	7.46	0.19	0.13	0.15	0.14	0.10	0.13	0.11
5	ρ-cymene	**1027**	7.70	0.35	0.18	0.36	0.38	0.18	0.35	0.31
6	Limonene	**1032**	7.84	0.59	0.45	0.46	0.41	0.27	0.40	0.41
7	γ-terpinene	**1062**	8.86	0.27	0.21	0.20	0.22	0.16	0.19	0.19
8	α-terpinolene	**1090**	9.91	0.18	0.14	0.11	0.13	0.10	0.10	0.10
9	n-heneicosane	**2100**	47.39	Tr	0.15	0.11	0.13	0.19	0.10	0.11
Oxygenated monoterpenes				**57.32**	**48.45**	**48.90**	**49.30**	**31.90**	**47.22**	**48.03**
10	1-octen-3-ol	**980**	6.29	0.92	0.56	0.88	0.92	0.24	0.81	0.78
11	1,8-cineole	**1034**	7.93	5.09	4.99	3.52	3.34	3.09	4.39	4.78
12	Linalool	**1101**	10.38	0.11	0.14	Tr	Tr	Nd	Tr	Tr
13	α-thujone (cis-thujone)	**1107**	10.61	28.34	21.61	26.40	27.49	13.09	26.19	25.05
14	β-thujone (trans-thujone)	**1119**	10.99	5.58	3.18	5.00	4.99	2.22	4.46	4.50
15	Camphor	**1147**	12.06	13.51	11.88	10.24	9.40	8.37	8.82	10.13
16	Borneol	**1168**	12.94	2.27	3.73	1.74	1.95	3.09	1.52	1.80
17	Terpinen-4-ol	**1179**	13.41	0.25	0.28	0.27	0.30	0.25	0.26	0.21
18	α-terpineol	**1192**	13.96	0.11	0.14	0.12	0.11	0.12	0.12	0.10
19	Cis-dihydrocarvone	**1198**	14.27	0.13	Nd	0.10	Tr	Nd	Nd	Nd
20	Bornyl acetate	**1287**	17.92	0.86	1.80	0.63	0.68	1.28	0.56	0.67
21	Trans-sabinyl acetate	**1293**	18.23	0.15	0.15	Nd	0.10	0.15	0.10	Nd
Phenolic monoterpenes				**0.93**	**1.24**	**2.77**	**1.25**	**0.99**	**0.88**	**1.16**
22	Thymol	**1295**	18.30	0.53	0.57	2.34	0.90	0.45	0.51	0.75
23	Carvacrol	**1304**	18.68	0.41	0.68	0.43	0.36	0.54	0.37	0.42
Sesquiterpene hydrocarbons				**10.03**	**13.80**	**8.10**	**8.52**	**13.69**	**9.06**	**10.89**

		RI								
24	Trans-caryophyllene	1419	23.40	2.84	4.48	1.37	1.73	2.89	1.56	2.41
25	α-humulene	1454	24.78	6.33	7.94	5.63	5.61	8.49	6.43	7.20
26	allo-aromadendrene	1461	25.06	0.12	0.14	0.11	0.11	0.14	0.11	0.11
27	α-selinene	1494	26.46	Tr	0.11	Tr	0.11	0.17	Tr	0.10
28	ð-cadinene	1524	27.61	Tr	Tr	Tr	Nd	0.12	Nd	Nd
29	α-trans-Bergamotyl acetate	1804	37.91	0.18	0.35	0.10	0.13	0.49	Tr	0.17
30	Longifolen	2083	46.87	0.57	0.78	0.88	0.83	1.39	0.97	0.90
Oxygenated sesquiterpene				**12.35**	**15.23**	**14.20**	**15.11**	**20.75**	**15.41**	**14.87**
31	Caryophyllene oxide	1582	29.83	0.31	0.28	0.21	0.29	0.33	0.30	0.30
32	Viridiflorol	1591	30.19	9.41	13.02	10.74	11.35	16.94	11.09	11.48
33	Carotol	1597	30.45	0.46	0.44	0.51	0.55	0.62	0.62	0.41
34	Humulene epoxide I	1601	30.60	0.11	0.18	0.12	0.13	0.20	0.23	0.10
35	α-Humulene epoxide II	1607	30.83	1.55	0.92	1.74	1.80	1.54	2.15	1.78
36	Humulene epoxide III	1628	31.57	0.15	0.13	0.17	0.17	0.20	0.24	0.14
37	Isospathulenol	1633	31.78	0.36	0.26	0.72	0.68	0.75	0.65	0.65
38	(E)-14-hydroxy-9-epi-caryophyllene	1660	32.76	Tr	Nd	Nd	0.16	0.18	0.12	Nd
Diterpene hydrocarbons				**0.09**	**0.15**	**0.15**	**0.15**	**0.25**	**0.14**	**0.15**
39	Rimuene (Rosa-5,15-diene)	1927	41.96	Tr	0.15	0.15	0.15	0.25	0.14	0.15
Oxygenated diterpene				**13.80**	**16.71**	**20.30**	**20.88**	**29.24**	**22.54**	**20.72**
40	Sclareoloxide	1906	41.30	0.12	0.19	0.18	0.20	0.30	0.22	0.18
41	manool	2053	45.92	13.57	16.40	19.98	20.53	28.13	22.32	20.38
42	Sclareol	2225	51.02	0.11	0.12	0.14	0.16	0.81	Nd	0.17
Notidentified compounds				**0.57**	**0.51**	**0.64**	**0.60**	**0.62**	**0.79**	**0.66**
43	Unknown	1869	40.02	0.40	0.24	0.64	0.60	0.41	0.79	0.66
44	Unknown	1780	37.05	0.18	0.27	Nd	Tr	0.21	Nd	Tr
Total (%)				**99.17**	**99.20**	**98.80**	**99.18**	**99.17**	**99.01**	**99.38**

[a] RI: Retention index on a HP-5 MS column; [b] Nd: Not detected; Tr: Traces <0.1%
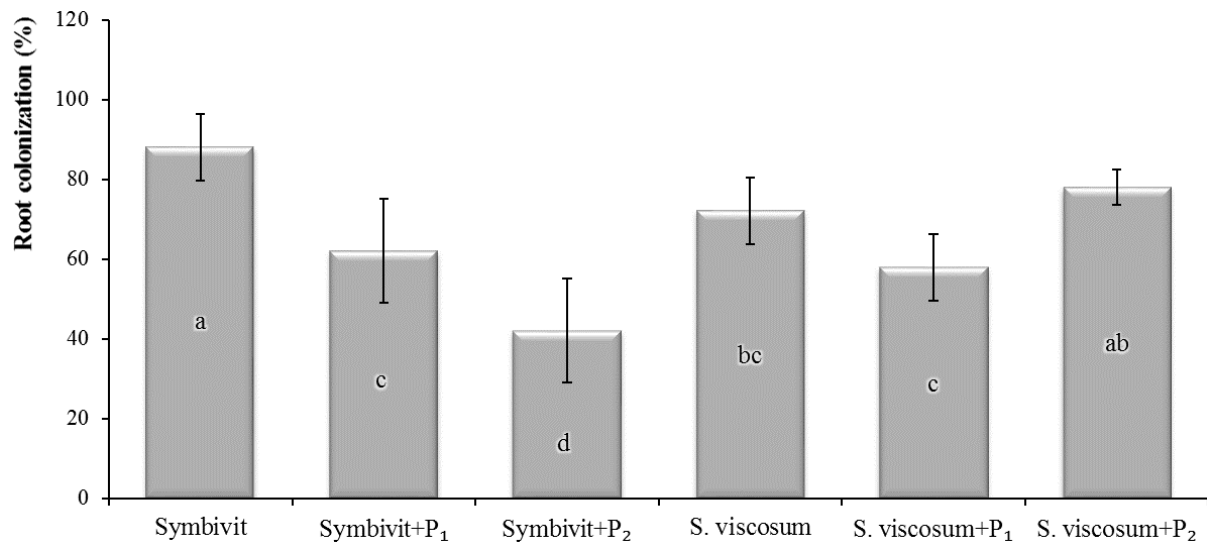
Figure

Fig. 1. Root colonization (percentages; mean±SD) of *Salvia officinalis* L. after treatment with P fertilization and mycorrhizal inoculation. Values with the same letters are not significantly different (P<0.05)