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Influence of arbuscular mycorrhizal fungi on growth, mineral composition and production of essential oil in *Mentha* × *piperita* L. var. *citrata* (Ehrh.) Briq. under two phosphorus levels

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The aim of the study was to evaluate the effect of the arbuscular mycorrhizal fungi (AMF), *Acaulospora morrowiae*, *Rhizophagus clarus* and *Scutellospora calospora* on the growth and essential oil production of *Mentha* × *piperita* L. var. *citrata* plants. The experiment was conducted in a greenhouse with a 5 × 2 factorial design with five mycorrhizal treatments (uninoculated control, *Acaulospora morrowiae*, *Rhizophagus clarus* and *Scutellospora calospora*, and mixture of inocula) and two P levels (60 and 120 mg P dm^{-3} soil) with six replicates. Plants were harvested after 75 days of growth for evaluation of dry biomass, mycorrhizal colonization of roots, number of spores in soil, nutrient content of leaves, and composition, content and yield of essential oil. The mycorrhizal treatments influenced all the characteristics evaluated, except the number of spores in soil. AMF colonization was most evident at the lowest dose of phosphate fertilizer applied, except for *R. clarus*, and in all treatments mycorrhizal colonization was above 45%. Colonization also influenced the nutrient content of the leaves of *M. piperita* var *citrata* and increased leaf dry biomass. The content and yield of essential oils were higher in plants colonized by AMF fungi with the 60 mg P fertilization dose, except those colonized by *R. clarus* which had higher essential oil production only with the 120 mg dm^{-3} P treatment. The two major components in the essential oils for the plants in all the treatments were linalool and linalyl acetate.

Key words: *Acaulospora, Rhizophagus*, *Scutellospora*, linalool, linalyl acetate, phosphorus fertilization.

INTRODUCTION

Mentha x *piperita* L. var. *citrata* (Ehrh.) Briq. belongs to the Lamiaceae family and the main components of its essential oils are linalool and linalyl acetate (Garlet et al., 2013). The presence of these compounds increases economic interest in this species, since they are used in the perfumery, cosmetics, food, pharmaceutical, fragrances

and tobacco industries, mainly as a flavoring (Steffani et al., 2006; Garlet et al., 2013). From a plant biology perspective, these compounds are associated with defense against pathogens and herbivores, and with attraction of pollinators (Croteau et al., 2000). The roots of plants in this genus form symbiotic associations with arbuscular mycorrhizal fungi (AMF) that in addition to their provision of mutual growth benefits can also influence the production of active components of the essential oils of *Mentha* spp. (Volpin et al., 1994; Morais, 2009; Arango et al., 2012).

The arbuscular mycorrhizal symbiosis has important ecological and biotechnological potential. Benefits are related to nutrient uptake from soils, especially those that have low mobility such as phosphorus, zinc and copper (Moreira and Siqueira, 2006). The improvement of host plant P nutrition has been recognized as one of the greatest benefits of mycorrhiza, but the results vary according to the level of P in the soil, with the species of AMF inoculated, and with plant species and/or cultivar (Cardoso et al., 2010).

Many studies have shown the efficiency of AMF inoculation in enhancing the development of essential oil producer plants and also their influence on the concentration of the main constituents of essential oils, as observed for *Mentha arvensis* L. (Gupta et al., 2002), *Coriandrum sativum* L. (Kapoor et al., 2002), *Baccharis trimera* (Less.) DC. (Freitas et al., 2004b), *Ocimum basilicum* L. var. Genovense (Copetta et al., 2006), *Origanum* sp. (Khaosaad et al., 2006), *Rosmarinus officinalis* L. and *Ocimum basilicum* L. (Russomano et al., 2008), *Inula ensifolia* L. (Zubek et al., 2010), *Artemisia umbelliformis* Lam. (Binet et al., 2011) and *Mentha piperita* L. (Arango et al., 2012).

The published scientific literature records a total of 47 studies that have focused on the association between arbuscular mycorrhizal fungi (AMF) and the production of secondary metabolites from medicinal plants (Zeng et al., 2013). In essential oil producer plants, AMF may influence the active components by improving plant nutritional status, or more directly as a response of the plant to fungal inoculation (Volpin et al., 1994; Morais, 2009). In *Origanum onites* and *Mentha viridis*, it has been reported that non-mycorrhizal plants had reduced levels of macro and micro nutrients in comparison to (for example) *O. onites,* which increased its P concentration after inoculation with *Glomus etunicatum* (Karagiannidis et al., 2011). In addition to AMF inoculation, the level of P in the soil also affected plant growth, for example, Arango et al. (2012) observed that mycorrhized *M. piperita* plants grown with 40 mg of phosphorus had higher P, K and Ca content in their shoots and had increased essential oil yield compared to those grown with 10 mg of phosphorus.

The aim of this study was to evaluate the effect of mycorrhizal fungi on plant growth, as well as on the content of macro- and micronutrients, yield and essential oil composition of *Mentha* × *piperita* var. *citrata* cultivated under two phosphorus levels.

MATERIALS AND METHODS

Experimental design, fungal inoculum and growth conditions

The experiment was conducted in a greenhouse at the State University of Santa Cruz, Ilhéus, Bahia, Brazil, under natural light and temperature. The experimental design consisted of a 5 × 2 factorial with three AMF inocula tested (gifted by Embrapa Agrobiology, Seropédica, RJ): *Acaulospora morrowiae* Spain and Schenck (A79 CNPAB 037), *Rhizophagus clarus* (Nicol. and Schenck) C Walker & A. Schüssler (A5 CNPAB 005) and *Scutellospora calospora* (Nicol. and Gerd.) Walk. and Sand. (A80 CNPAB 038), a mixture of all three AMF species, and a control consisting of uninoculated plants.

Two phosphorus levels (60 and 120 mg dm^{-3}) were applied as single superphosphate [$Ca(H_2PO_4)_2$. H_2O,$CaSO_4.H_2O$] in soil with ten repetitions at each level. Half of the plant samples were used for growth evaluation and other half for analyses of essential oil production.

Cuttings of *M.* × *piperita* var. *citrata* (approx. 5 cm in length) were obtained from the Universidade Estadual de Santa Cruz (UESC) Garden of Medicinal Plants, and were rooted for 14 days in a sand and vermiculite (2:1) substrate that was autoclaved prior to use. At the time of transplant, plants were inoculated with AMF spores in pots containing 4 dm^3 of soil which had the following chemical and physical characteristics (Embrapa, 1997): pH ($CaCl_2$) 4.0; organic matter 17 g dm^{-3}; P < 2 mg dm^{-3}; K = 0.4 mmolc dm^{-3}; Ca = 5 mmolc dm^{-3}; Mg = 3 mmolc dm^{-3}; B = 0.49 mg dm^{-3}; Cu < 0.3 mg dm^{-3}; Fe = 171 mg dm^{-3}; Mn = 2.0 mg dm^{-3}; Zn = 0.9 mg dm^{-3}; Al = 5.4; H + Al = 47 mmolc dm^{-3}; SB = 7.9 mmolc dm^{-3}; CTC = 54.8 mmolc dm^{-3}; V(%) 14; coarse sand = 142 g kg^{-1}, fine sand = 720 g kg^{-1}; silt = 13 g kg^{-1} clay and 125 g kg^{-1}. Prior to plant transplantation, the soil was limed (pH adjusted to 6.5 using 11.42 g limestone dm^{-3} soil) and fertilized based upon a soil analysis, estimated plant productivity and taking into consideration AMF inoculation, and was comprised of: two doses of P (60 and 120 mg dm^{-3}), 100 mg dm^{-3} N in the form of $(NH_4)2SO_4$, 80 mg dm^{-3} K (KCl), 1.55 mg dm^{-3} Fe (EDTA-Fe), 0.75 mg dm^{-3} B (boric acid), 1.33 mg dm^{-3} Cu ($CuSO_4$ $5H_2O$), 0.15 mg dm^{-3} Mo [$(NH_4)_6Mo_7O_{24}$ $4H_2O$], 4 mg dm^{-3} Zn and Mn ($ZnSO_4$ $7H_2O$ and $MnSO_4$, respectively). The soil was autoclaved for 1 h at 121°C and at 1.5 atm for two consecutive days to sterilize it.

Plant harvests and determination of biomass

After 75 days of growth in the greenhouse, plants were harvested, separated into roots, stems and leaves of these three were dried at 70°C and then weighed for determination of biomass. This same material was used to analyze the nutrient content.

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Table 1. ANOVA results (two-way factorial analysis) for the effects of AMF accessions, P dose and interaction on *Mentha x piperita* var. *citrata* traits.

Trait	AMF accessions			P dose			AMF × P dose		
	df	F	P	Df	F	P	df	F	P
Leaf dry weight	4	2.682	0.061	2	3.591	0.072	4	15.279	0.000
Leaf P concentration	4	6.948	0.001	2	27.718	0.000	4	1.207	0.342
Leaf K concentration	4	5.935	0.003	2	9.813	0.00	4	4.067	0.016
Leaf S concentration	4	3.377	0.031	2	0.101	0.754	4	2.649	0.067
Leaf Ca concentration	4	2.044	0.131	2	4.510	0.047	4	2.512	0.078
Leaf Mg concentration	4	2.632	0.068	2	0.366	0.55	4	1.355	0.288
Leaf Fe concentration	4	2.077	0.122	2	4.827	0.040	4	2.007	0.132
Leaf Cu concentration	4	2.297	0.094	2	13.005	0.001	4	5.786	0.002
Leaf Mn concentration	4	3.498	0.025	2	0.660	0.426	4	8.375	0.000
Mycorrhiza colonization	4	56.040	0.000	2	9.798	0.005	4	10.494	0.000
Essential oil content	4	5.026	0.005	2	3.199	0.088	4	4.191	0.012
Essential oil yield	4	8.872	0.000	2	0.237	0.631	4	15.027	0.000

Factors were: AMF accessions (*Acaulospora morrowiae* - A79 CNPAB 037, *Rhizophagus clarus* - A5 CNPAB 005 and *Scutellospora calospora* - A80 CNPAB 038) and P dose (60 and 120 mg dm^{-3} soil).

Nutrient content

For determination of nutrient content, about 0.20 g of the leaves were weighed into glass tubes, to which were then added 3 ml of concentrated HNO_3, and these were then transferred to a digester block for 30 min at 50°C, and then for 90 min at 120°C. Final digestion was performed with three consecutive additions of 1 ml of 30% H_2O_2 which were maintained at 120°C for 20 min each. The resulting solution was transferred to 15 ml centrifuge tubes in which the volume was made up to 12 ml with ultrapure water. The solutions were then read in an optical spectrophotometer (ICP AOS) Variant 710-ES.

Percentage of mycorrhizal colonization

The percentage of mycorrhizal colonization (Mcgonigle et al., 1990) was determined in triplicate from random samples. Vertical stripes were made on blades and roots placed horizontally and the intersection between the stripe and the root of the presence or absence of mycorrhiza inoculation and type of structure detected was observed. For this, the roots were clarified and stained according to a modified version of the method used for Phillips and Hayman (1970). First, the roots were cleared with 10% potassium hydroxide solution in a water bath at approximately 60°C for 30 min, and then with 10% hydrogen peroxide for 20 min at room temperature. Finally, the roots were rinsed with distilled water and stained in an acidophilic dye based on black ink and 5% acetic acid. The determination of the number of AMF spores was carried out in triplicate using a modified version of the wet sieving method of Gerdemann and Nicolson (1963), using a Leica EZ4 stereomicroscope at 40× magnification. Soil samples were centrifuged twice, the first time with distilled water for 3 min at 2500 rpm, and then with 50% sucrose for 1 min at 2500 rpm.

Content, yield and essential oil composition

For essential oil evaluation another three replications (from leaves) were collected and dried at 40°C. The extraction of essential oils was performed using a Clevenger apparatus with 2 to 3.6 g of dry leaves in 400 ml of water for one hour, with three replications per treatment from a sample composed of all replicates for each treatment.

The content of the oil was determined based on the weight of oil extracted from dried plant material (% m/m) in an analytical balance. The oil yield was calculated by dividing the content multiplied by the average leaf dry biomass (mg $plant^{-1}$). The oils were chemically characterized according to their refractive index (RI), and later by gas chromatography coupled to a high-resolution mass spectrometer (HRGC). The various chemical constituents of the essential oils were identified by comparing computed with the library unit, literature and by Kovats retention index (Adams, 1995). The Kovats retention indices (KI) were calculated by the injection of a series of n-alkane standards (C8-C26) injected under the same chromatographic conditions as the samples.

Statistical analysis

All data were subjected to analysis of variance, with phosphate treatments compared by F-test at 5% probability. For means of AMF treatments, a Scott-Knott test at 5% probability using Sisvar software was used (Ferreira, 2000). The graphs were constructed using the Statistica software (StatSoft, Tulsa, OK).

RESULTS

Biomass production

There were interactions between the factors evaluated and the variables analyzed (Table 1). With 60 mg dm^{-3} added P a higher leaf biomass of *M. piperita* was observed for plants inoculated with a mixed inoculum,

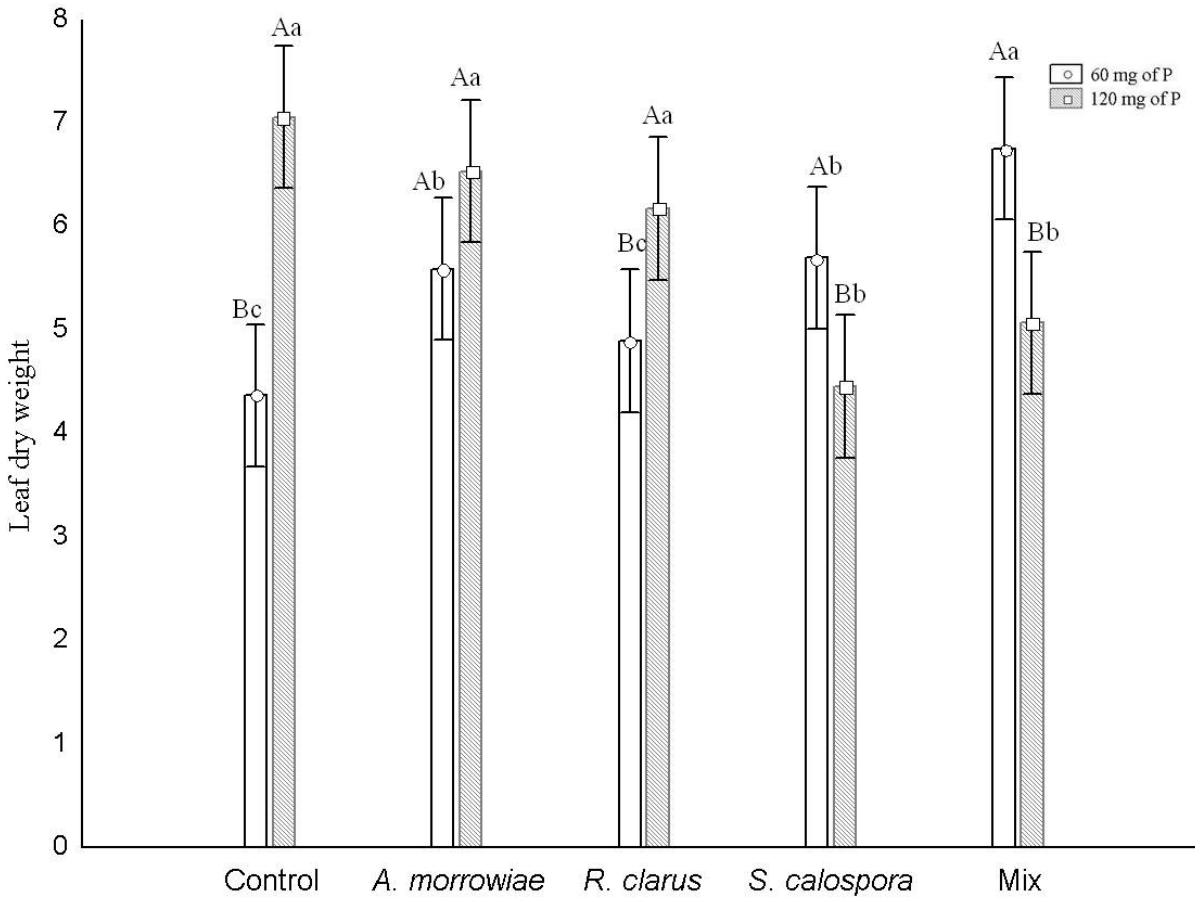

Figure 1. Leaf dry weight (mg) of *Mentha* x *piperita* var. *citrata* plants inoculated with three AMF species and mix fertilized with two P doses.

and with 120 mg dm^{-3} added P higher leaf production was observed on plants inoculated with uninoculated plants, *A. morrowiae* and *R. clarus*. When analyzing the effect of P rate on AMF treatments, we found that only inoculation with *A. morrowiae* showed no significant difference. Furthermore, on uninoculated plants and those inoculated with *R. clarus*, leaf biomass production was directly proportional to the two P doses. The treatments with inoculation of *S. calospora* and a mixture of inocula resulted in biomass production greater than that achieved with 60 mg P (Figure 1).

Nutrient content

We observed that only Zn showed no interaction between the factors or any significance in results in at least one of the factors (Table 1). With 60 mg dm^{-3} P, plants inoculated with *A. morrowiae*, the mixed inoculum and the control treatments had significantly higher S contents than those inoculated with *R. clarus* and *S. calospora*. Plants inoculated with *S. calospora* had higher concentrations of Ca and Mg in their leaves, whereas for P concentration the plants with the mixed inoculum significantly differed from the other treatments. Leaves from the mixed inoculum plants showed greatest concentration of P. Higher concentrations of Fe and Cu were obtained in leaves of *S. calospora* and in the AMF mixed inoculum plants (Table 2). Application of 120 mg dm^{-3} P demonstrated a less generalized effect on nutrients, since only the P content in plants from all the treatments and K in the mixed inoculum plants differed significantly from the uninoculated control plants. On the other hand, inoculation with AMF (particularly the mixed inoculum) decreased Mn content compared to plants from the other treatments (Table 2).

When the effect of superphosphate fertilization level was evaluated for each AMF treatment we observed that the Ca concentration in the plants with the mixed inoculum differed significantly, since the higher P dose

Table 2. Concentration of macronutrients (g kg^{-1}) and micronutrients (mg kg^{-1}) in the leaves of *Mentha* x *piperita* var. *citrata* plants inoculated with three AMF species or with a mixture of all three when fertilized with two P doses.

P doses (mg)	Control	*A. morrowiae*	*R. clarus*	*S. calospora*	Mix
			P		
60	1.58^{Ab}	1.85^{Bb}	1.74^{Bb}	1.79^{Bb}	2.28^{Aa}
120	1.83^{Ab}	2.36^{Aa}	2.54^{Aa}	2.35^{Aa}	2.59^{Aa}
			K		
60	31.52^{Aa}	30.27^{Aa}	28.31^{Ba}	32.07^{Aa}	32.57^{Ba}
120	32.37^{Ab}	33.08^{Ab}	33.04^{Ab}	27.97^{Ac}	39.52^{Aa}
			S		
60	11.21^{Aa}	11.11^{Aa}	6.31^{Bb}	8.59^{Ab}	11.85^{Aa}
120	7.24^{Ba}	12.39^{Aa}	10.06^{Aa}	9.50^{Aa}	11.39^{Aa}
			Ca		
60	8.62^{Ab}	8.70^{Ab}	9.30^{Ab}	11.46^{Aa}	9.41^{Bb}
120	8.95^{Aa}	10.56^{Aa}	10.68^{Aa}	9.41^{Aa}	11.45^{Aa}
			Mg		
60	4.89^{Ab}	5.21^{Ab}	5.25^{Ab}	6.94^{Aa}	5.45^{Ab}
120	5.05^{Aa}	6.06^{Aa}	4.94^{Aa}	5.82^{Aa}	6.36^{Aa}
			Fe		
60	111.33^{Ab}	132.00^{Ab}	132.66^{Ab}	171.00^{Aa}	149.66^{Aa}
120	122.66^{Aa}	126.00^{Aa}	121.00^{Aa}	122.00^{Ba}	127.66^{Aa}
			Zn		
60	43.57^{Aa}	42.01^{Aa}	55.94^{Aa}	45.76^{Aa}	46.86^{Aa}
120	43.55^{Aa}	40.75^{Aa}	47.64^{Aa}	40.76^{Aa}	43.29^{Aa}
			Cu		
60	8.56^{Ab}	8.01^{Ab}	7.72^{Ab}	10.62^{Aa}	11.74^{Aa}
120	7.88^{Aa}	7.97^{Aa}	8.67^{Aa}	6.66^{Ba}	7.82^{Ba}
			Mn		
60	229.66^{Ba}	261.33^{Aa}	224.66^{Aa}	252.66^{Aa}	266.00^{Aa}
120	302.66^{Aa}	254.66^{Ab}	233.00^{Ac}	229.33^{Ac}	178.33^{Bd}

Means (n = 3) followed by the same uppercase letter in the columns and lowercase letter in the rows for each content of micronutrients belong to the same group by Scott-Knott test at 5% of probability.

(120 mg dm^{-3}) applied resulted in a higher Ca concentration and the opposite occurred with the Mn concentration (Table 2). For leaf P-content the superphosphate fertilization level influenced *R. clarus*, *S. calospora* and *A. morrowiae*-inoculated plants, but not those with the mixed inoculum or the uninoculated plants (Table 2). For leaf K-content the higher superphosphate level resulted in the *R. clarus*-inoculated and the mixed inoculum plants differing significantly from those with the lower superphosphate level with these two AMF treatments. For Fe-content, the *S. calospora*-inoculated plants grown with 60 mg of P were higher than those from the other treatments (Table 2).

Mycorrhizal colonization

For the percentage of mycorrhizal colonization on *M. piperita* var. *citrata* plants each AMF species inoculated gave different results; with the 60 mg dm^{-3} dose of phosphorus the root colonization percentage was highest in *S. calospora*, followed by *A. morrowiae*, and then the mixed inoculum plants, but at the 120 mg dm^{-3} P level the *R. clarus*-inoculated plants had a higher percentage of mycorrhizal colonization (Figure 2). For all AMF treatments, except for the *R. clarus*-inoculated plants with the higher superphosphate fertilization, the plants had reduced levels of mycorrhizal colonization in their roots

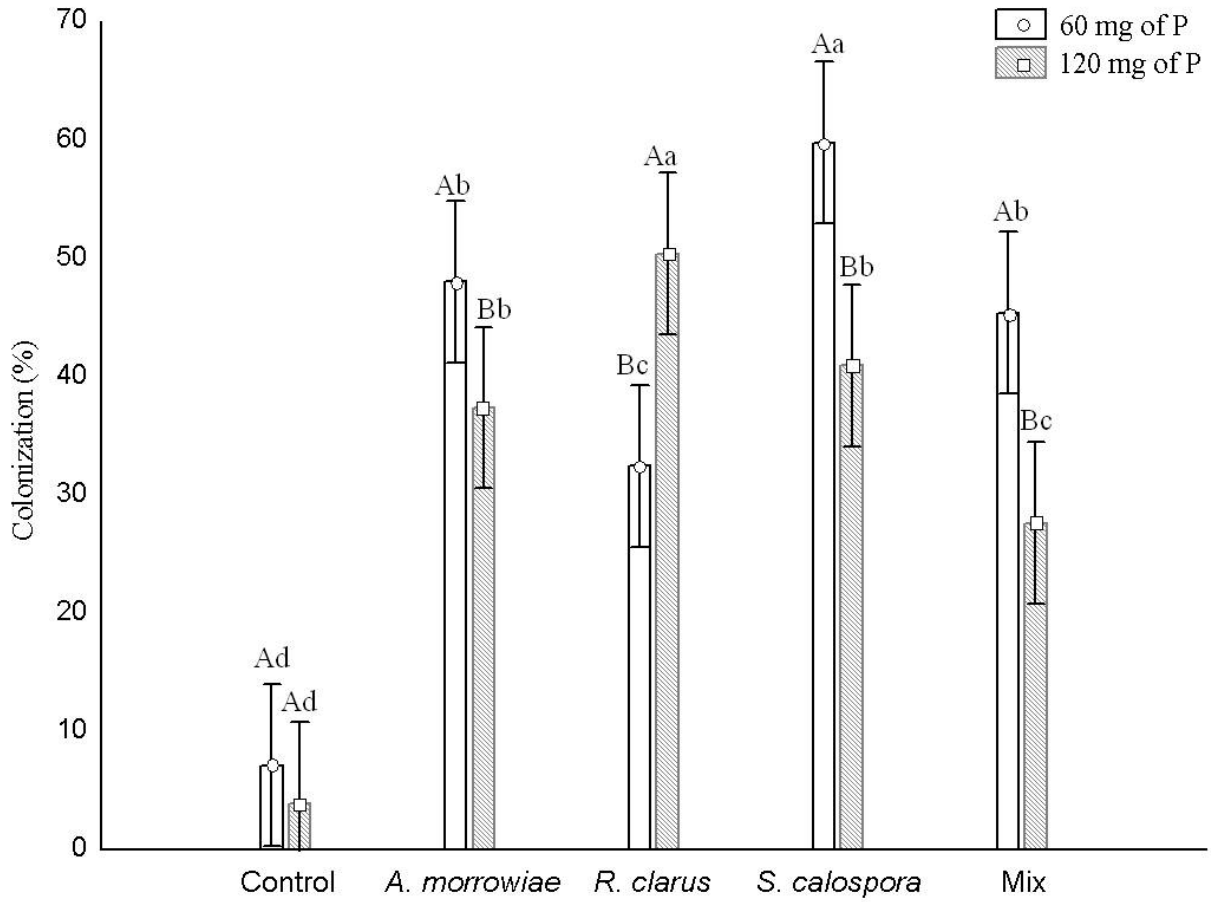

Figure 2. Root mycorrhizal colonization (%) of *Mentha* x *piperita* var. *citrata* plants inoculated with three AMF species and mix fertilized with two P doses.

(Figure 2). Arbuscules were the most abundant structures observed in all AMF treatments, regardless of applied P level, indicating that all three fungal species formed functional symbioses with *M. piperita* var. *citrata* plants (data not showed). The *A. morrowiae*-inoculated plants had higher number of arbuscules compared to the other treatments at the lower P level. At the higher dose of superphosphate the *R. clarus*- inoculated plants had significantly higher numbers of arbuscules compared to the other treatments. Typical AMF structures, such as arbuscules, vesicles and spores were observed in *M. piperita* var. *citrata* roots (Figure 5).

Content, yield and essential oil composition

At the 60 mg dm^{-3} P level, the essential oil content and yield of AMF inoculated-*M. piperita* var. *citrata* plants were significantly higher than those of the control plants (Figure 3). At the 120 mg dm^{-3} P dose inoculation with *R. clarus* gave the highest content and yield of essential oil extracted from the leaves. The analysis of the effect of P-level on each fungus showed differences with those plants inoculated with *A. morrowiae* and *S. calospora* for their essential oil content. For the yield of essential oils differences appeared for all treatments, with the exception of the *A. morrowiae*-inoculated plants. The lower level of phosphate fertilization resulted in higher essential oil yields for the *S. calospora*-inoculated and mixed inoculum plants (Figure 4). We identified thirteen essential oil components in *M.* × *piperita* var. *citrata* and only two organic compounds were not determined. The major components for all treatments were linalool and linalyl acetate. In addition, nine other components were identified, but with reduced percentages, including nerol, neryl acetate, geranyl acetate and caryophyllene (Table 3). At the lowest dose of phosphate fertilization the leaves of plants inoculated with *R. clarus* gave the

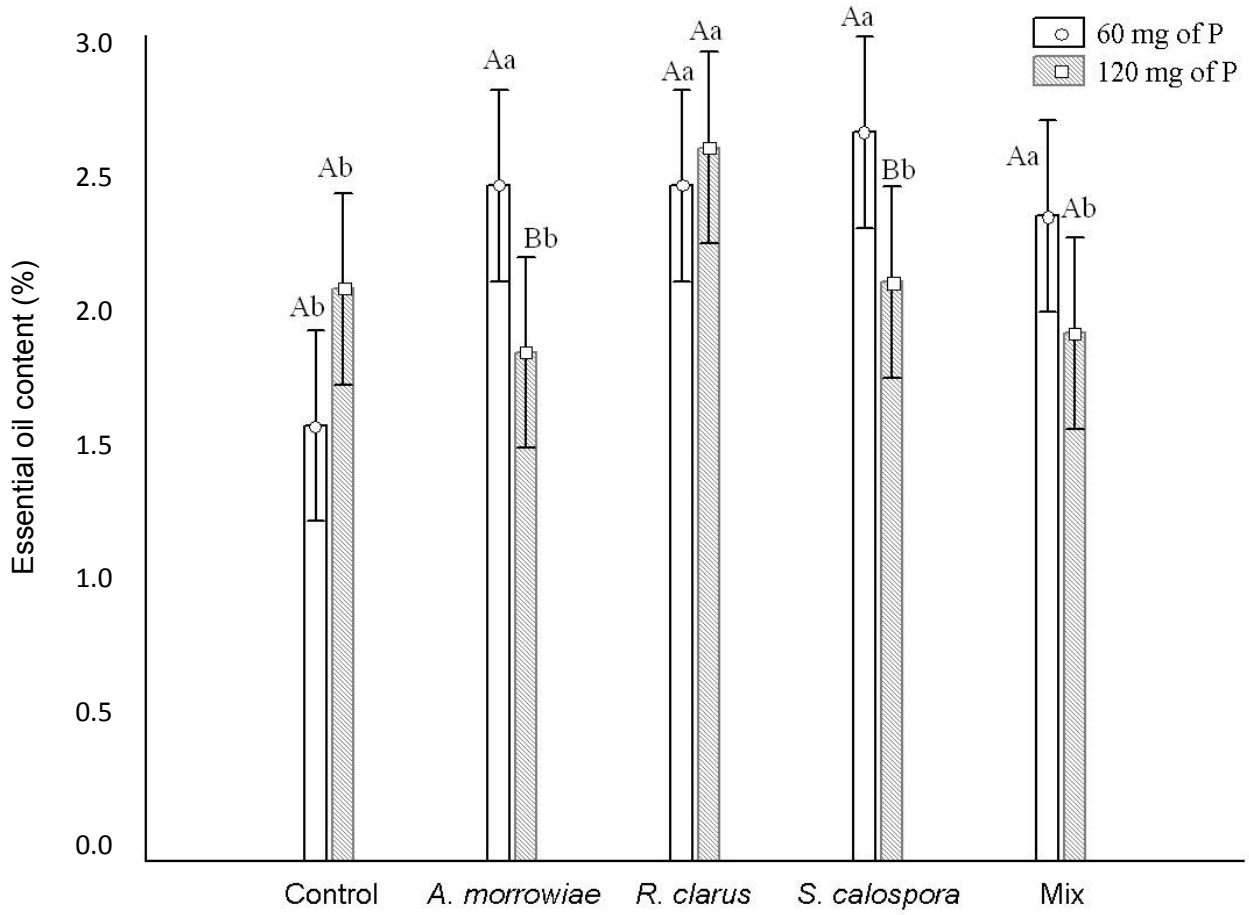

Figure 3. Content (%) of essential oil of *Mentha* x *piperita* var. *citrata* plants inoculated with three AMF species and mix fertilized with two P doses.

highest percentage of linalool (51%) and the lowest percentage of linalyl acetate (24.5%). At the higher dose of phosphate fertilization the *S. calospora*-inoculated plants obtained the highest percentage of linalool (48%) and the *R. clarus*-inoculated plants had the highest percentage (37%) of linalyl acetate.

DISCUSSION

A high input of P fertilizer in soil (120 mg kg^{-1} P) did not promote significant increases in leaf biomass of mycorrhized *M*. × *piperita* var. *citrata*, except for plants inoculated with *A. morrowiae* and *R. clarus*. In addition, a high superphosphate input also did not result in an increase in essential oil content in leaves of *M*. × *piperita* var. *citrata*. The increases in plant biomass were particularly demonstrated under low P availability, as observed with *M. piperita* fertilized with 10 and 40 mg P kg^{-1} soil (Arango et al., 2013), and this was corroborated in our study for *M*. × *piperita* var. *citrata* plants supplied with low P levels (60 mg kg^{-1} P). Biomass increases are probably a result of the improved nutritional status of mycorrhizal plants, as the extracellular hyphae of the fungi greatly expand the effective surface of the plant in contact with the soil, colonizing sites not previously explored by the roots, and thus optimizing nutrient uptake (Clark and Zeto, 2000). This probably occurred on mycorrhized *M*. × *piperita* var. *citrata* plants since leaf nutrient concentrations were higher than those of uninoculated plants.

Higher P level (120 mg dm^{-3}) clearly depressed leaf production and reduced the essential oil content of *M*. *piperita* var. *citrata* when inoculated with *S. calospora*, probably due to decreasing of mycorrhizal colonization. In addition, the pH of the soil was adjusted to 6.5 in our experiment, and this may have reduced mycorrhizal colonization as *S. calospora* seems to be adapted to

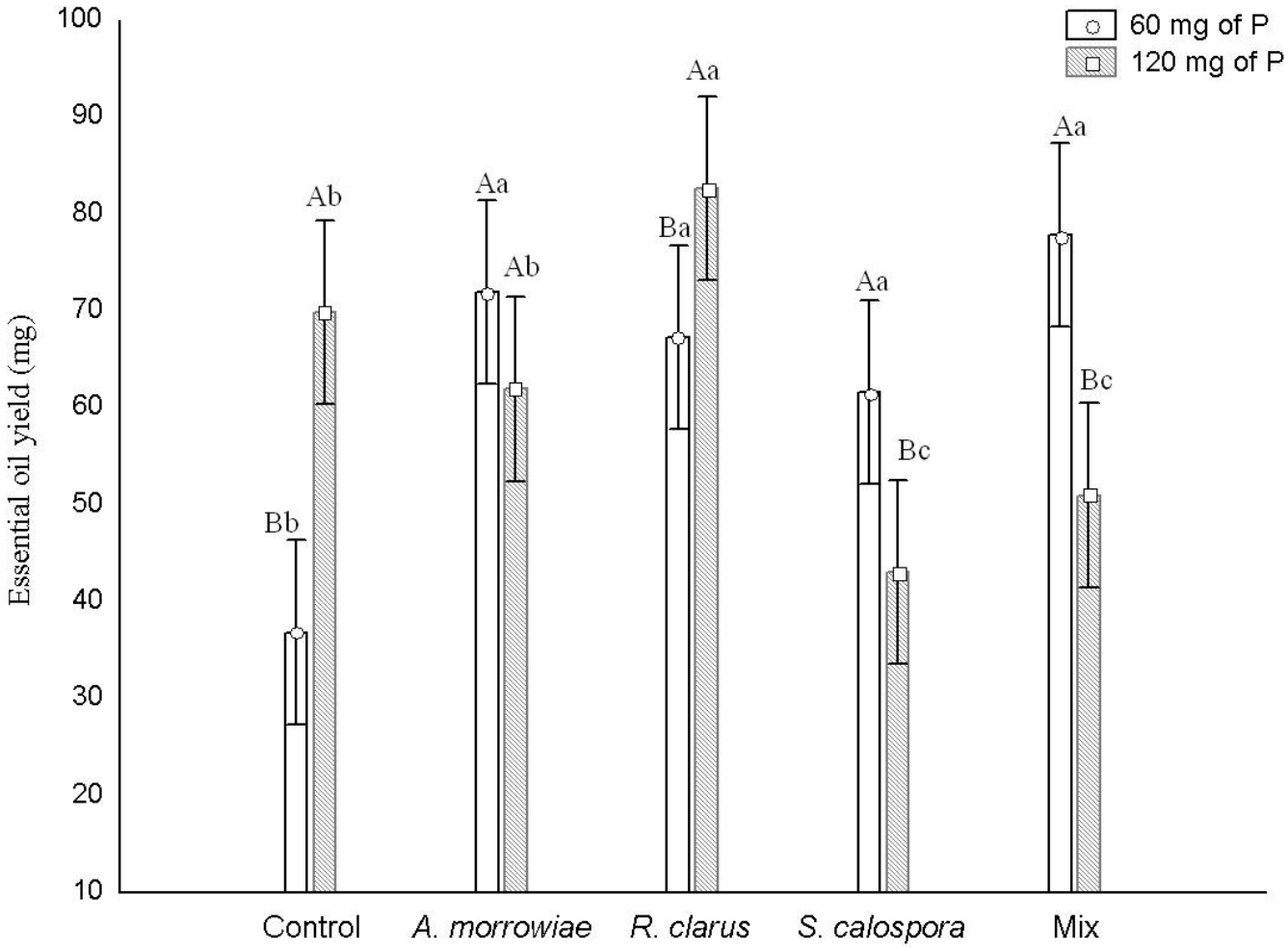

Figure 4. Yield (mg) of essential oil of *Mentha* × *piperita* var. *citrata* plants inoculated with three AMF species and mix fertilized with two P doses.

more acidic soils (Dickson et al., 1999; Manjarrez et al., 2008), and this may also explain some of the low levels of macro and micronutrients observed in *M*. *piperita* var. *citrata* leaves. Another possibility is low compatibility between the plant and fungus (Johnson et al., 1997), which is based on the absence of a growth response due to the costs of symbiosis. In this case, the low P uptake from the soil via the AMF hyphae and the high demand for carbohydrates by the fungus can result in a net drain of resources, and hence reduced plant growth. Indeed, it is known that the uptake and transfer of P by AMF is highly variable according to fungal species (Smith et al., 2011).

Soil nutrient concentrations, particularly, after phosphorus fertilization, can reduce mycorrhizal colonization of roots, but different fungal and plant species can respond differentially (Copetta et al., 2006; Freitas et al., 2006). This seems to have occurred with *M. piperita* var. *citrata* plants inoculated with *R. clarus* as this fungal species produced higher AMF colonization percentages, and increased nutrient and biomass content compared to other treatments at the higher phosphorus level added to the soil. The increase in phosphorus concentration in soil may inhibit the incidence of colonization, and one possible explanation is that the phosphorus content can modulate the activity of some enzymes involved in plant resistance to infection, thus decreasing mycorrhizal colonization (Lambais and Mehdy, 1993).

The plants of *M. piperita* var. *citrata* inoculated with *S. calospora* and supplied with low P levels had adequate nutritional status with high macro and micronutrient contents. This probably was influenced by the high percentage of mycorrhizal colonization which positively

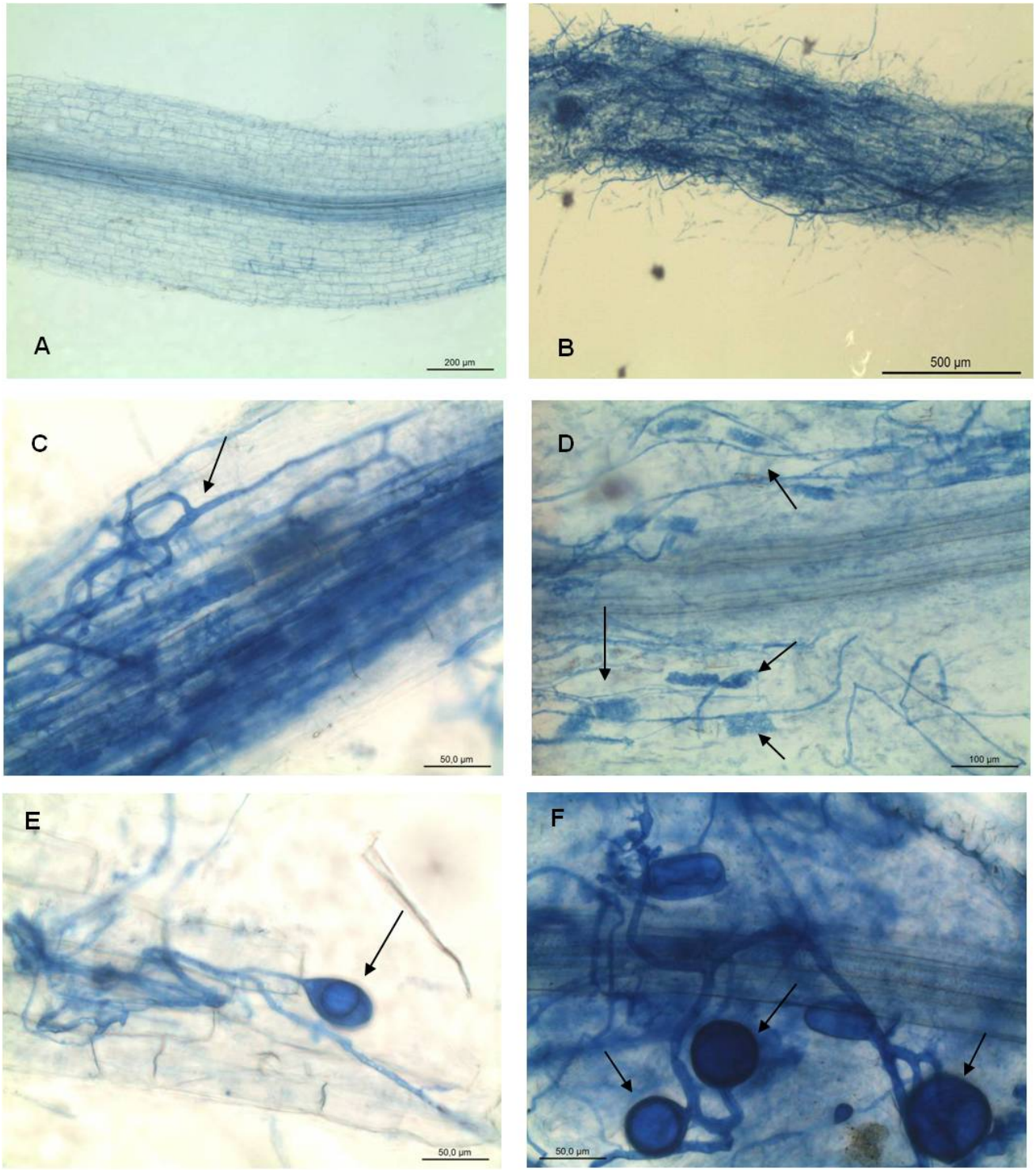

Figure 5. Images of roots stained by the method of Phillips and Haymann showing the morphology of mycorrhiza in *Mentha* × *piperita* var. *citrata* from low supply P level (60 mg dm^{-3} soil) plants. (A) General view of a root segment without AMF colonization from a control (uninoculated) plant. (B) Root segment of highly AMF-colonized root from a mixed inoculated plant. (C) Detail of a root segment with intra-and extra-cellular hyphae and arbuscles (arrowhead) from a mixed inoculated plant. (D) Arbuscules (arrows) highly branched inside cortical cells of a *Scutelospora calospora*-inoculated plant. (E) Vesicle (arrowhead) and hyphae colonizing a root segment from a mixed inoculated plant. (F) Oblong vesicles (arrowheads) and a ticked wall spore (arrow) of a *Scutelospora calospora*-inoculated plant.

Table 3. Kovats indices (IK) and percentage of the chemical constituents of the essential oil of the biomass of the leaves of *Mentha x piperita* var. *citrata* (E.) Brinq. inoculated with three AMF species and fertilized with two P doses.

Constituents	IK*	P Doses									
		60 mg dm^{-3}					120 mg dm^{-3}				
		C	Am	Rc	Sc	M	C	Am	Rc	Sc	M
Linalool	1100	41.06	48.44	51.9	47.37	46.9	42.52	41.55	46.24	48.06	46.77
Cis-3-pinanone	1175	-	0.56	0.58	0.64	0.48	0.45	0.41	0.53	0.41	0.37
α- terpineol	1191	5.65	5.16	6.24	3.99	4.07	4.85	5.06	3.85	4.04	4.42
Nerol	1227	1.94	1.27	1.39	0.88	0.94	1.16	1.31	0.89	0.96	1.15
Linalyl Acetate	1259	25.33	29.37	24.56	35.50	35.53	36.03	34.44	37.25	34.01	33.86
ND**	-	6.93	4.03	4.35	2.66	2.95	3.65	4.23	2.77	2.94	3.67
Neryl Acetate	1363	1.85	1.50	1.61	1.12	1.10	1.36	1.49	1.05	1.11	1.23
Geranyl Acetate	1382	4.42	3.18	3.35	2.32	2.29	2.90	3.20	2.16	2.35	2.64
Caryophyllene	1419	1.25	1.36	1.37	1.67	1.43	1.69	1.57	1.47	1.47	1.28
γ- gurjuneno	1479	1.01	0.84	0.72	0.86	0.75	0.95	0.97	0.76	0.81	0.75
Elemol	1546	3.16	1.79	1.55	0.89	1.16	1.54	2.46	0.98	1.38	1.24
ND**	-	0.68	-	-	-	-	-	-	-	-	-
Guaiol	1590	6.68	2.43	2.33	2.05	2.35	2.85	3.25	2.00	2.39	2.56
Total identified (%)	-	99.96	99.93	99.95	99.95	99.95	99.96	99.93	99.95	99.95	99.94

*IK obtained by capillary column VF5-ms (30 m × 0.25 mm × 0.25 μm). temperature of the injector and detector 250 and 280°C. respectively; **ND = Not Determined; = uninoculated control; Am = *Acaulospora morrowiae*; Rc = *Rhizophagus clarus*; Sc = *Scutellospora calospora*; M = mixed inoculum

affected leaf biomass production and essential oil content since these traits can be directly affected by increased nutrient uptake promoted by AMF mainly in soils with low levels of available P (Nasiri et al., 2010; Karagiannidis et al., 2011; Mandal at al., 2013).

The plants inoculated with *A. morrowiae* and supplied with high P levels also had adequate nutritional status and high macronutrient concentrations in leaves, and this was reflected in their increased leaf biomass production, but not in their essential oil content, and their percentage of mycorrhizal colonization was decreased. In contrast, plants inoculated *R. clarus* under high P levels had moderate nutrient contents and leaf biomass production, but with high essential oil content and the highest percentage of root colonization. These two AMF species gave different results when inoculated onto *M. piperita* var. *citrata*, as did the plants treated with the mixed AMF inoculum which had their highest mycorrhizal colonization and leaf essential oil content with the low P level despite only having moderate nutrient concentrations in their leaves. Different AMF species or accessions produce differential growth and nutritional status that affect essential oil production on aromatic or medicinal plants (Copetta et al., 2006; Freitas et al., 2006; Toussaint et al., 2007; Chaudhary at al., 2008; Karagiannidis et al., 2012).

There is an involvement of putative direct mechanisms of AMF stimuli (Gupta et al., 2002; Kapoor et al., 2002; Freitas et al., 2004a; Toussaint et al., 2007), such as phytohormones (Copetta et al., 2006; Toussaint et al., 2007) and/or carbohydrate reallocation towards isoterpenoid synthesis (Mandal et al., 2013; Asensio et al., 2012) for essential oil production. Our results reinforced this idea taking into account that high P levels (120 mg) promoted a higher leaf biomass production on uninoculated plants but just a slight, disproportional increase in essential oil content when compared with uninoculated low P-fertilized plants. For producing and increasing essential oil in plants, phosphorus is required as an integral constituent of the precursor glyceraldehyde 3-phosphate in the plastidial MEP (methylerythritol) pathway and of the intermediate mevalonate phosphate in the cytoplasmic MVA (mevalonate) pathway (Mandal et al., 2013), as well as the pyrophosphate intermediary compounds in the terpene synthetic pathway (Kapoor et al., 2004; Mandal et al., 2013).

The purpose of research on AMF inoculation in medicinal or essential oil-producing plants has been not only to focus on increasing essential oil production but also to improve the oil quality (Zeng et al., 2013). Many of the publications reported no qualitative changes in

essential oil composition, but a quantitative change in the percentage of some components, as occurred with inoculated *M. piperita* var. *citrata* in our study. This was observed, for example for *Ocimum basilicum* L. var. *Genovense* using *Gigaspora rosea* and *G. margarita* (Copetta et al., 2007), for *Inula ensifolia* that showed variation in content of thymol and your derivatives when inoculated with two strains of *Glomus intraradices* and one of *R. clarus* (Zubek et al., 2010). In mint and oregano inoculated with two strains of *Glomus etunicatum* and *G. lamellosum*, changes also occurred in major components of essential oil (Karagiannidis et al., 2011). In the present study, the major components of *M. piperita* var. *citrata* for all treatments were linalool and linalyl acetate, confirming observations reported in the literature (Garlet et al., 2013), where *M*. × *piperita* var. *citrata* is considered as a promising species for the production of linalool.

In addition to the influence of AMF, phosphorus fertilization also has a strong influence on the content, yield and composition of essential oils, as demonstrated by our results with *M*. × *piperita* var. *citrata* cultivated under two P levels, and also previously observed by (Freitas et al., 2004a) with AMF-inoculated *M. arvensis* and who showed that in the absence of phosphate fertilization there were changes in the major compound of essential oil in this plant.

Conclusion

Several studies have demonstrated that mycorrhizal inoculation increases plant biomass production, and in medicinal and essential oil-producing plants increases the essential oil content. The frequency and intensity that this occurs depends largely on the plant - fungus interaction and growth conditions. Our study demonstrated that essential oil production in *M. piperita* var. *citrata* plants can be achieved with the use of half doses of P combined with inoculation with effective AMF accessions, and that these can also improve essential oil quality and content, and reduce production costs. For this purpose, a 60 mg kg^{-1} of P fertilization plus inoculation with *Scutellospora calospora* (A80 CNPAB 038) is recommended. Our results also provided evidence that increasing essential oil content in *M. piperita* var. *citrata* leaves is mediated by direct and indirect effects of AMF inoculation.

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Conflict of interest

The authors have declared that there is no conflict of interests.

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