

## Response of three mint and two oregano species to *Glomus etunicatum* inoculation

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### Abstract

The effect of *Glomus etunicatum* on root colonization, growth, essential oil content and composition and nutrient acquisition of *Oreganum vulgare*, *O. onites*, *Mentha viridis*, *M. spicata* and *M. piperata* was investigated. The results showed that inoculated plants had significantly higher shoot and root dry weight, nutrient concentration and total essential oil production compared to non-inoculated plants. The *Oreganum* species showed significant higher percentages of colonization than *Mentha* species. Analysis of essential oil by GC and GC/MS showed that the effect of *G. etunicatum* on the main volatile compounds in leaf essential oils was different on the plant species tested.

These results suggest that the use of *G. etunicatum* may enhance plant growth in low fertility soils, reduce fertilizer inputs and increase aromatic plant production of essential oils, while they indicate that it may be possible to use mycorrhizae to affect the quality of the essential oil produced.

**Keywords:** Colonization, Essential Oils, *Glomus etunicatum*, Growth, *Mentha*, Nutrient, *Oreganum*.

**Abbreviations:** AMF-Arbuscular mycorrhizal fungi; MS-mass spectrum; Co-GC-coinjection with authentic compound.

### Introduction

*Mentha* and *Origanum* are genera of flowering plants in the family Lamiaceae (mint family). Both are perennial medicinal herbs, native to warm-temperate western and southwestern Eurasia and the Mediterranean region. *Origanum* is used as a painkiller and anti-inflammatory. *Origanum* tea is a treatment for indigestion, coughs, and to stimulate menstruation. The oil of *Origanum* is used for toothache, and in some cosmetics. Its leaves and flowering stems are natural antiseptics because of high thymol content. Several *Mentha* species are considered industrial crops as they are a source of essential oils enriched in certain monoterpenes, widely used in food, flavourings, cosmetics and pharmaceutical industries. *Mentha* and *Origanum* have a large number of species that differ widely in their characteristics and polyploidy level (Bhat et al., 2002). *Origanum vulgare*, *O. onites*, *M. piperata*, *M. spicata*, and *M. viridis* predominate in Greek habitats. AMF are common forms of symbiotic fungi that form association with plant roots in a host non-specific manner (Shaul et al., 1990), colonize cortical tissues and extend hyphae into the rhizosphere (Hetrick et al., 1996). The phytobiont occurs in approximately 80% of plant species and the fungi are classified in the phylum Glomeromycota (Dalpe, 2004). Mycorrhizal fungi are considered to have many roles in nature and managed ecosystem (Bilalis et al., 2011). Mycorrhizae facilitate nutrient uptake especially phosphorus, nutrient recycling, water absorption, production of essential

oils, plant growth, increased tolerance to adverse soil pH, high temperature, drought and toxic heavy metals (Singh, 2003). Many studies have been done by various workers on the role of AMF in medicinal plants (Devi and Staramaiah, 1998; Martins et al., 2004; Olsson et al., 1999; Pandey and Banik, 2009; Silveira et al., 2006;). However, little is known about their potential effect on the quantitative and qualitative profile of the secondary metabolites (e.g., essential oils) in medicinal and aromatic plants (Copetta et al., 2006; Kapoor et al., 2002a; Morone-Fortunato and Avato, 2008). The objective of this paper is a comparative analysis of the effects induced by *Glomus etunicatum* on plant growth, nutrient uptake and essential oil content and composition of *O. vulgare*, *O. onites*, *M. piperata*, *M. spicata*, and *M. viridis* plants.

### Results

#### Root colonization

In the mycorrhizal treatments, all *Origanum* and *Mentha* plants were well colonized with the percentage of root colonization levels as follow: *O. vulgare* 86%, *O. onites* 85%, *M. viridis* 72%, *M. piperata* 68%, and *M. spicata* 65% (Table 1). The colonization of *Origanum* plants was significant higher than *Mentha* plants.

**Table 1.** Dry weight, essential oil content, and percentage of root colonization of *Oreganum vulgare*, *Oreganum onites*, *Mentha piperata*, *Mentha spicata*, and *Mentha viridis* plants inoculated with the indigenous *Glomus etunicatum*

Plant Species		Dry Weight <sup>y,z</sup> (mg)	Essential Oils (%)	Colonization (%)
<i>Mentha viridis</i>	M <sup>x</sup>	20.73 <sup>c</sup>	2.12 <sup>b</sup>	72 <sup>b</sup>
	NM	8.98 <sup>d</sup>	1.02 <sup>c</sup>	
<i>Mentha spicata</i>	M	32.72 <sup>b</sup>	2.24 <sup>b</sup>	65 <sup>b</sup>
	NM	9.34 <sup>d</sup>	1.39 <sup>c</sup>	
<i>Mentha piperata</i>	M	31.83 <sup>b</sup>	1.63 <sup>bc</sup>	68 <sup>b</sup>
	NM	9.19 <sup>d</sup>	0.86 <sup>c</sup>	
<i>Oreganum onites</i>	M	33.59 <sup>b</sup>	3.00 <sup>a</sup>	85 <sup>a</sup>
	NM	8.57 <sup>d</sup>	1.95 <sup>b</sup>	
<i>Oreganum vulgare</i>	M	41.52 <sup>a</sup>	3.35 <sup>a</sup>	86 <sup>a</sup>
	NM	8.16 <sup>d</sup>	1.68 <sup>bc</sup>	

<sup>x</sup>M = Mycorrhizal Plants, NM = Non-mycorrhizal plants, <sup>y</sup>Values are the means of 2 experiments, each with 4 replicates; results were similar according to the Bartlett's test of homogeneity of variance, so data were combined, <sup>z</sup>Values in the same column followed by different letters are significantly different ( $P = 0.05$ ) according to Duncan's Multiple Range Test.

#### Effect of *Glomus etunicatum* on plant growth and nutrient uptake

The highest dry weight was found in inoculated *O. vulgare* plants. The inoculated plants of *O. onites*, *M. piperata*, and *M. spicata* had similar dry weight which was significant higher than *M. viridis*. Similar dry weight was found in the non-inoculated plants of all *Oreganum* and *Mentha* species used which was significant lower than the inoculated *M. viridis* plants (Table 1). Inoculated plants of all *Oreganum* and *Mentha* species had similar concentrations in N, B, Zn, Cu and Mn, significantly higher than the non-inoculated plants (Table 2). The inoculated *O. vulgare*, *O. onites*, *M. piperata* and *M. spicata* plants had similar P concentrations, significantly higher than inoculated *M. viridis* plants. The non-inoculated plants of all *Oreganum* and *Mentha* species had similar P concentrations, significantly lower than the inoculated *M. viridis* plants.

The inoculated *O. vulgare* plants had the highest K concentration, while the non-inoculated plants of all *Oreganum* and *Mentha* species had the lowest. Similar K concentrations were found in inoculated plants of *O. onites*, *M. piperata*, *M. spicata* and *M. viridis* plants. The inoculated *M. viridis* plants had the highest Na concentration, while the non-inoculated plants of all *Oreganum* and *Mentha* species the lowest. Similar Na concentrations were found in inoculated plants of *O. onites*, *M. piperata*, *M. spicata* and *O. vulgare* plants. The highest Ca concentration was found in inoculated *O. vulgare* plants, while the non-inoculated plants of all *Oreganum* and *Mentha* species had the lowest. The inoculated *O. onites* plants had significant higher Ca concentration than the inoculated *M. piperata*, *M. spicata* and *M. viridis* plants. No significant difference in Ca concentrations was found among the non-inoculated plants of all *Oreganum* and *Mentha*. The inoculated *M. viridis*, *M. piperata* and *O. vulgare* plants had the highest Mg concentration, while the non-inoculated plants of all *Oreganum* and *Mentha* species the lowest. No significant difference was found in Mg concentration between *M. spicata* and *O. onites* (Table 2). The inoculated *M. piperata* showed the highest Fe concentration, while the non-inoculated plants of all *Oreganum* and *Mentha* species the lowest. No significant difference in Fe concentration was found between inoculated *O. vulgare* and *M. spicata*, which

was significant higher than inoculated *M. viridis* and *O. onites* plants (Table 2).

#### Effect of *Glomus etunicatum* on essential oil content and composition

The inoculated *O. vulgare*, and *O. onites* plants had the highest total essential oil production. Similar total essential oil production was found in inoculated *M. spicata*, *M. piperata*, *M. viridis* and non-inoculated *O. onites* and *O. vulgare* plants. No significant difference was found in total essential oil production among the inoculated *M. piperata* and the non-inoculated *M. viridis*, *M. spicata*, *M. piperata* and *O. vulgare* plants (Table 1). The results showed that there was an effect of the *G. etunicatum* on the composition and level of volatile compounds for all plant species (supplementary data). The chemical composition of essential oils differed in all plant species. The compounds p-Cymene, and  $\gamma$ -Terpinene were high in *O. vulgare* plants, higher in mycorrhizal plants. The compound Thymol was also high in *O. vulgare* plants, but was higher in non-mycorrhizal plants. High concentration of the Limonene was found in the *M. viridis* and *M. spicata* plants, higher in the mycorrhizal plants. The compound trans-Sabinene hydrate was high in *O. onites* plants, higher in mycorrhizal plants. The compound Linalool was high in *M. piperata* and *O. onites* plants, higher in non-mycorrhizal plants. The concentration of the compound Carvone was high in *M. viridis* (higher in nonmycorrhizal plants) and *M. spicata* plants (higher in mycorrhizal plants). The compound Linalyl acetate was high in *M. piperata* plants (higher in mycorrhizal plants). Finally, the concentration of the Carvacol was high in *O. onites* and *O. vulgare* plants, higher in non-mycorrhizal plants (supplementary data).

#### Discussion

In all treatments, mycorrhizal plants had increased growth, nutrient contents and total essential oil production (Table 1). Generally, *Oreganum* plants had higher percentage of colonization than *Mentha* plants. The positive effect of AM-related root colonization by *G. mosseae* on the increase of the shoot biomass of oregano plants grown in a greenhouse has

**Table 2.** Nutrient concentration in the shoots of *Oreganum vulgare*, *Oreganum onites*, *Mentha piperata*, *Mentha spicata*, and *Mentha viridis* plants inoculated with the indigenous *Glomus etunicatum*

Plant Species		N%	P%	K%	Na%	Ca%	Mg%	B (mg l <sup>-1</sup> )	Zn (mg l <sup>-1</sup> )	Mn (mg l <sup>-1</sup> )	Fe (mg l <sup>-1</sup> )	Cu (mg l <sup>-1</sup> )											
<i>Mentha viridis</i>	M <sup>x</sup>	2.49 <sup>y</sup>	a <sup>z</sup>	0.39	b	3.75	b	5.07	a	1.60	c	0.86	a	68.63	a	95.66	a	144.07	a	602.77	c	88.17	a
	NM	0.88	b	0.13	c	1.19	c	2.07	c	0.82	e	0.27	c	24.10	b	29.97	b	21.90	b	126.80	d	12.60	b
<i>Mentha spicata</i>	M	2.54	a	0.75	a	3.81	b	3.97	b	1.82	c	0.59	b	71.80	a	140.67	a	142.00	a	732.50	b	92.40	a
	NM	0.76	b	0.15	c	1.21	c	2.20	c	0.68	e	0.33	c	26.50	b	27.20	b	20.23	b	126.30	d	13.20	b
<i>Mentha piperata</i>	M	2.84	a	0.73	a	3.40	b	3.37	b	1.64	c	0.98	a	67.07	a	135.40	a	139.10	a	919.10	a	80.70	a
	NM	0.71	b	0.12	c	1.18	c	1.70	c	0.72	e	0.33	c	22.90	b	16.90	b	21.73	b	122.10	d	11.40	b
<i>Oreganum onites</i>	M	2.53	a	0.66	a	3.35	b	3.73	b	2.43	b	0.65	b	83.67	a	134.57	a	151.47	a	508.93	c	108.10	a
	NM	0.69	b	0.10	c	1.17	c	1.83	c	0.51	e	0.30	c	19.20	b	21.67	b	18.70	b	136.50	d	9.51	b
<i>Oreganum vulgare</i>	M	2.38	a	0.77	a	5.73	a	3.00	b	3.91	a	0.96	a	82.97	a	133.57	a	131.10	a	756.70	b	114.57	a
	NM	0.79	b	0.13	c	1.17	c	1.90	c	0.52	e	0.28	c	24.77	b	20.87	b	23.23	b	94.87	d	10.83	b

<sup>x</sup>M=mycorrhizal plants

NM=non-mycorrhizal plants

<sup>y</sup>Values are the means of 2 experiments, each with 4 replicates; results were similar according to the Bartlett's test of homogeneity of variance, so data were combined

<sup>z</sup>Values in the same column followed by different letters are significantly different ( $P = 0.05$ ) according to Duncan's Multiple Range Test.

been reported by Khaosaad et al. (2006). The effects of colonization by mycorrhizal fungi on growth, nutrient contents and total essential oils have also been reported for many other plants, such as *Ocimum basilicum* (Copetta et al., 2006; Rasouli-Sadaghiani et al., 2010), red clover (Sturmer, 2004). Other work has shown that root colonization by AMF results in a positive effect on plant growth, mainly through an improved P status of the host plant (Smith and Read, 1997). Khaosaad et al. (2008) confirmed that inoculation of red clover plants with *Glomus mosseae* stimulated the growth and the P content. The higher nutrient content in AMF inoculated plants is attributed to higher influx of nutrients into the plant system through AM fungi which explore the soil volume beyond depletion zone as previous works have shown for P (Bayaraj and Varma, 1995; Sanders and Tinker, 1971). According to Bagyaraj and Reddy (2000), the extramatrical hyphae produced by AM fungi act as extensions of roots and increase the surface area of the root system, making it more efficient for absorption of water and diffusion of limited nutrients, this effect being more pronounced in P-deficient soils. Previous works have shown that AMF increase plant uptake of phosphate (Bolan, 1991), micronutrients (Burkert and Robson, 1994), nitrogen (Barea et al., 1991). Moreover, it has been demonstrated that plants inoculated with AMF utilize more soluble phosphate from rock phosphate than noninoculated plants (Antunes and Cardoso, 1991). Similarly, the results of this study have shown that inoculated plants of *O. vulgare*, *O. onites*, *M. viridis*, *M. spicata* and *M. piperata* plants had higher nutrient contents in comparison to non-inoculated (Table 2). This study also showed that mycorrhizal plants had higher total essential oil production than non-mycorrhizal (Table 1). *Oreganum vulgare* and *O. onites* inoculated with *Glomus etunicatum* seems to have better response in the total essential oils production than the *Mentha* species used in this study. Morone-Fortunato and Avato (2008) found that inoculation of *O. vulgare* plants with the mycorrhizal fungus *G. viscosum* improved the plant biomass and provided a better qualitatively and quantitatively profile of essential oils. In contrast, inoculation of *O. vulgare* with *G. mosseae* did not alter the concentration of the essential oils (Khaosaad et al., 2006) leading to the conclusion that different fungi can modulate the yield of essential oils differently in the same plant. Analysis of essential oil by GC and GC/MS showed that the effect of *G. etunicatum* on the main compounds in leaf essential oils was different on the plant species used (supplementary data). Some studies have been previously carried out to investigate the effect of mycorrhizal inoculation on the quantity and quality of essential oils in aromatic plants. Thus, the concentration of volatiles and the relative amount of trans-anethole was improved by AM inoculation of *Foeniculum vulgare* (Kapoor et al., 2004). The application of mycorrhizal fungi, also had a positive effect on the amount of oils and content of the main metabolites in *Anethum graveolens* (Kapoor et al., 2002b), *Mentha arvensis* (Gupta et al., 2002), *Coriandrum sativum* (Kapoor et al., 2002a) and *Trachyspermum ammi* (Kapoor et al., 2002b). In other studies, Copetta et al. (2006) compared different AM fungi inoculation on basil and found that AM fungi induced various modifications in the considered parameters. Geneva et al. (2010) found that the *G. intraradices* inoculation of

*Salvia officinalis* in combination with fertilization promoted the production of 1,8-cineole and alpha-thujone, mycorrhizal colonization enhanced bornyl acetate, 1,8-cineole, alpha- and beta-thujones. The mechanism behind changes in essential oil composition is not known and it is possible that it may be related to better nutrition. However, Khaosaad et al. (2006) concluded that the quantitative increase in oregano essential oil with mycorrhizae was not due to improved P nutrition. Generally, all oregano and mint plants, besides an increased plant growth, have a higher content of essential oils and nutrient elements when mycorrhizal. Mycorrhization, in mint and oregano plants, can also change the essential oil composition. These results indicate the importance of the AM symbiosis, that should be managed to help in the reduction of fertiliser and other agrochemical inputs, thus enhancing the sustainability of the commercial cultivation of aromatic plants, even in low fertility, mountainous soils.

## Materials and methods

### Isolate of *Glomus etunicatum*

A strain of *G. etunicatum*, isolated from oregano plant collected from Ritini, Pieria, Greece, was used in this study. Identification was made by using the molecular method described by Karagiannidis et al. (2011) and maintained on maize mother plants.

### Plant culture and mycorrhizal colonization

Plants of *O. vulgare*, *O. onites*, *M. piperata*, *M. spicata*, and *M. viridis* were propagated by stem cuttings (originated from the mother plantations established in the experimental field of Alexander Technological Education Institute of Thessaloniki) rooted in autoclaved perlite in the greenhouse under a misting system. The rooted cuttings were transplanted in April in pots containing 1.5 kg of soil substrate mixed with fine cut pieces of highly colonized roots of maize plants with a pure *G. etunicatum* strain following the method described by Karagiannidis (1980). For the mycorrhizal treatments, 10 cm<sup>3</sup> inoculum per pot (about 12g) was placed in the planting hole beneath the plants at transplanting. The inoculum consisted of colonized root fragments (roots were cut into 1cm pieces), hyphae and spores. Pots without inoculum were used as control. The soil used was a sandy loam with a pH 6.5; low salinity (0.82 mS conductivity), low organic matter (0.6%), low CaCO<sub>3</sub> (0.11%) with a Ca, Mg and P content at 152, 9.6, and 0.7 mg/kg respectively and it was autoclaved at 120 °C for 25 minutes. A fertilization treatment with 25.4 mg kg<sup>-1</sup> KCl, 29.7 mg kg<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>, 162 mg kg<sup>-1</sup> Ca<sub>5</sub>(PO<sub>4</sub>)<sub>3</sub>OH, 27.6 mg kg<sup>-1</sup> MgCl<sub>2</sub>.6H<sub>2</sub>O and 190 mg kg<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub> was added to the potting mix before planting. A total of 200 pots were used with the following combinations: 2 inoculation treatments (inoculated plants with *G. etunicatum*; non-inoculated control) replicated 4 times of 5 pots for each of the plant host species evaluated. Plants were grown for 2.5 months in the greenhouse. Shoots and roots were harvested separately to analyse nutrient and essential oil composition in the shoots and the establishment of the arbuscular mycorrhizal symbiosis in the roots. The shoots were air-dried for three days (10% moisture content) and nutrients were analysed

after mineralization by calcination in a muffle furnace at 450°C for 8 hours, and extraction with HCl (35 %). Na and K were determined using flame photometry. N was determined using the Kjeldahl method, P was determined using chromatography and Ca and Mg were determined volumetrically using the Versenate method. Concentration of trace elements was determined with atomic absorption spectrophotometry (Fe, Mn, Cu, Zn) and B with the azomethine. All methods are described in Jackson (1960) and Cottenie (1980). Roots were stained with trypan blue (Sylvia, 1994) and mycorrhizal colonization was estimated according to McGonigle et al. (1990).

#### ***Isolation and analysis of the essential oils***

Leaves were separated from shoots, and the essential oils were obtained by hydrodistillation in 500 mL H<sub>2</sub>O in a Clevenger apparatus for 2 hours. The composition of the volatile constituents was established by GC and GC-MS analyses. GC analyses were performed on a Shimadzu GC-14A, with a FID (Flame Ionization Detector), using a DB5 column (30 m x 0.25 mm, film thickness: 0.25µm). The temperature program was from 65°C for 10 min, to 160°C at a rate of 3 °C/min for 5 min. Helium was used as a carrier gas at a flow rate of 0.6 ml/min. GC-MS analyses were performed on a Shimadzu GC-2010–GCMS-QP2010 system operating in EI mode (70eV) equipped with a split/splitless injector (230°C), a split ratio 1/30, using a fused silica HP-5 MS capillary column (30 m x 0.25 mm (i.d.), film thickness: 0.25 µm) and a polar column HP-Innowax. The analytical conditions were: for the HP-5MS column the temperature program was from 60°C (5min) to 280°C at a rate of 4°C /min and for the HP-Innowax column the temperature program was from 60°C (5min) to 260°C at a rate of 3°C /min. Helium was used as a carrier gas at a flow rate of 0.8 ml/min. Injection volume of each sample was 1 µl. Retention indices for all compounds were determined using n-alkanes as standards. The identification of the components was based on comparison of their MS with those of NIST21 and NIST107 and those described by Adams (2001). Quantitative determination was based on the total ion count detected by the GC-MS. This experiment was repeated.

#### ***Statistical analysis***

Plants were cultured in a completely randomized block design with 4 replicates of 5 pots for each treatment. Statistical analysis of the data was carried out by using analysis of variance. Means were separated by using the Duncan's multiple range test at 0.05 significance level.

#### ***Conclusion***

Our findings showed that the commercial value of the aromatic and medicinal plants can be improved by using mycorrhizal plants increasing the income of the growers. In addition, the use of AM fungi possibly makes the commercial cultivation of aromatic and medicinal plants economically viable even in low fertility soils.

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