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ORIGINAL PAPER

Plant development and synthesis of essential oils in micropropagated and mycorrhiza inoculated plants of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart

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Abstract Biomass production of micropropagated oregano was induced by inoculation with the fungus *Glomus viscosum.* The effects of arbuscular mycorrhizal (AM) symbiosis on morphological and metabolic variations of regenerated oregano plants were investigated at different growth stages. AM greatly increased parameters such as plant leaf area, fresh and dry weight, number of spicasters and verticillasters in infected plants. An increase of the gland density, especially on the upper leaf epidermis, was also observed following the physiological ageing of the tissues. The in vitro plants of *O. vulgare* ssp. *hirtum* described in this study provided a qualitatively and quantitatively good source of essential oils that have a chemical profile comparable to that of the control mother plants with carvacrol as the main compound.

Keywords *Origanum*; *O. vulgare* ssp. *hirtum* · Oregano · Micropropagation · Mycorrhiza · Essential oils · Secretory glands · Carvacrol

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Introduction

Plant members within the *Origanum* taxa produce essential oils which differ both in terms of quantity and quality (Kokkini 1996; Skoula and Harborne 2002). *Origanum vulgare* ssp. *hirtum* (Lamiaceae) appears particularly rich in volatiles, mainly phenolic compounds such as carvacrol and/or thymol (D'Antuono et al. 2000; Fleisher and Sneer 1982; Kokkini 1996; Russo et al. 1998; Skoula and Harborne 2002). Secretion of the essential oils is associated with the presence of glandular hairs on the plant aerial parts, consisting of two different types, peltate and capitate trichomes (Bosabalidis 2002; Morone-Fortunato and Circella 1993). Among the two types of glands generally found in the Lamiaceae, the capitate hairs may synthesize metabolic products other than essential oils, for example mucilage; sessile peltate hairs are reported to be exclusively involved in the secretion of the essential oils (Bosabalidis 2002). They are described as consisting of a 12-celled head, a unicellular stalk, a unicellular base and a peribasal crown of 12–18 radial epidermal cells.

Oregano has long been recognized as a culinary herb and medicinal plant (Mäkinen and Pääkkönen 2002; Baricevic and Bartol 2002) with beneficial effects on the digestive and respiratory systems and antiseptic, antispasmodic, carminative and cholagogue properties (Duke, http://www.ars-grin.gov/duke/). Several breeding programmes have been undertaken to improve oregano production and the yield of its active compounds

(Franz and Novak 2002; Makri 2002). However, attempts to increase the amount of highly valuable metabolites by applying biotechnological methods for the propagation of this plant appear to be rather limited (Kintzios 2002; Baricevic 1997). To the best of our knowledge, the in vitro production of essential oils has only been studied in micropropagated plants of *O. bastetanum* (Socorro et al. 1998) and in calli from *O. vulgare* ssp. *virens* (Alves-Pereira and Fernandes-Ferreira 1998), while the synthesis of rosmarinic acid has been investigated in shoot cultures of *O. vulgare* (Yang and Shetty 1998).

Secondary metabolites are believed to play an important role in plant–mycorrhiza interaction and it has been shown that in certain plants, fungus symbiosis induces changes in the root accumulation of secondary compounds, some of them acting as signal molecules (Akiyama and Hayashi 2002; Peipp et al. 1997; Smith et al. 2006). Although there is enormous data on the yield and growth increase in several ornamental and vegetable crops as well as trees, limited research has been conducted to investigate the contribution of such microorganisms on the quantitative and qualitative profile of the secondary metabolites typical of the plant (Strack et al. 2003; Xin et al. 2006). In particular, only a few studies have been carried out to establish the role of arbuscular mycorrhizal (AM) in enhancing the productivity of essential oil bearing plants (Copetta et al. 2006; Gupta et al. 2002; Kapoor et al. 2002a; Kapoor et al. 2002b; Kapoor et al. 2004; Khaosaad et al. 2006; Ueno and Shetty 1998). A recent study (Khaosaad et al. 2006) on the effect mycorrhization has on oregano showed that colonization clearly increases the amount of shoot biomass and moreover, essential oil yields were variable.

In an attempt to understand the effects on the physiology of micropropagated *O. vulgare* ssp. *hirtum* and on the metabolism of its volatile oils, studies were carried out on the influence of the mycorrhizal inoculation on micropropagated plant growth, development of secretory structures and production of essential oils. As far as we know, such a study has never been performed previously on *O. vulgare* ssp. *hirtum*.

Materials and methods

Plant material

An *O. vulgare* L. ssp. *hirtum* (Link) Ietswaart (syn. *O. heracleoticum* L. and *O. megastachyum* Link) genotype from a 2-year-old oregano crop from the field collection of medicinal plants at the Faculty of Agriculture of the University of Bari (Italy) was used as source plant material for the experiments in this study. Plant samples were collected at different physiological stages and identified in the text as follows: control mother plants: 3-month-old cuttings (MP3), 6-month-old cuttings (MP6), 12-month-old cuttings at the flowering stage (MP). Micropropagated plants: 3-month-old plants during acclimatization (VA), 6-month-old plants growing outdoors (VF), 12-month-old plants growing outdoors at the flowering stage (VFB). Parallel mycorrhization experiments were conducted with mother plants as well as with micropropagated oregano. Plants treated with the fungus are referred to as Myc^+, while Myc^- stands for the control non-inoculated plants.

Micropropagation

Axillary buds, 4–5 mm length from the *O. vulgare* L. ssp. *hirtum* genotype were used as explants to establish micropropagated clones. Buds were surface sterilized in a 20% commercial sodium hypochlorite (4.9 % active chlorine) solution for 20 min. Plant preparations were successively rinsed with sterile water.

The basic culture medium (BM), containing the macronutrients according to Murashige and Skoog (1962), the micronutrients of Nitsch and Nitsch (1969), FeEDTA (25 mg l^{-1}), thiamine HCl (0.4 mg l^{-1}), myoinositol (100 mg l^{-1}), sucrose (20 mgl^{-1}), agar (7 gl^{-1}) was employed for in vitro cultures. The pH of the medium was adjusted to 5.6–5.8. Sterilization of culture media was performed in autoclave at 121°C for 20 min; the culture of the explants was carried out under a horizontal laminar flow hood to ensure the necessary sterile conditions. During the trial, oregano explants were maintained in a growth chamber at 24 ± 1°C with a photoperiod of 16 h light under a light intensity of 30 μE m^{-2} s^{-1} (Morone-Fortunato et al. 2006).

Primary explants were cultured on BM enriched with 6-benzylaminopurine (BAP) at 0.5, 1.0, 1.5, 2.0 mg l^{-1}. Plant tissue culture tubes (20 × 150 mm; Sigma) containing 20 ml BM were inoculated with the primary explants. A total of 80 primary explants were cultured in each of the BAP concentrations. The efficacy of the BAP concentration was determined by recording the percentage of primary explant growth,

the rate of vitrification, browning and contamination, the number of developing shoots and the length and number of microcuttings per shoot. Uninodal microcuttings (5–6 mm), obtained from 5 week old shoots grown on BM supplemented with 1 mg l^{-1} BAP were subcultured twice at intervals of about 3 weeks using the same medium conditions. A rooting phase was not necessary, as during the second subculture, nearly 90% of the microcuttings showed root growth. Microplants were transferred to 10 cm diameter pots filled with a commercial peat mixture (organic carbon 23%, organic nitrogen 0.4%, organic matter 80%, Gebr. Brill Substrate GmbH & Co.K.G. D-49828 Georgsdorf. RFG) and an addition of perlite (2:1, v/v). Acclimatization took place in greenhouse conditions of 15–18°C using a misting-bed system in which the humidity level was reduced from 85–90% to 55–60% over 20–25 days.

The chromosome number, peltate trichome density and essential oil production was calculated for micropropagated (VFB) and control mother plants (MP) at the flowering stage.

Cuttings

The same genotype as above was selected as the source of cuttings. Rooted cuttings were transplanted into 10 cm diameter pots. The potting media and acclimatization procedures were the same as for micropropagated plants (see above). Potting media sterilization was made in autoclave for 20 min at 121°C.

Field

After acclimatization (late summer, September), microplants and cuttings were transferred outdoors (Agriculture Research Station, University of Bari, South Italy). They were planted at 50 and 40 cm distance between rows and within row, respectively. Irrigation was ensured only when necessary.

Mycorrhization

Before acclimatization, the roots of micropropagated plants and cuttings were inoculated with the arbuscular mychorrizal (AM) fungus *Glomus viscosum* T.H. Nicolson, strain A6 (kindly provided by Prof. M. Giovannetti, Università, Pisa). Ten g of crude AM inoculum were added to each pot to infect micropropagated plants and cuttings. Crude AM inoculum consisted of infested soil containing fungus spores, mycelium hyphae and infected root fragments from strawberry pot-cultures inoculated with the same A6 strain of *Glomus viscosum*. The same growth conditions were applied to Myc^+ plants as those for the non-inoculated plants (see above). After 3 months, Myc^+, Myc^- micropropagated plants and the control mother cuttings were transferred to 1,000 ml pots (one plant per pot) containing the commercial peat mixture (see above). A total of 50 plants for each treatment (Myc^- and Myc^+ micropropagated and the control mother plants) were prepared and grown in a random design. Plants were transferred outdoors with irrigation applied when needed.

The analysis of the colonized roots was carried out according to Phillips and Hayman (1970) with the samples observed under an optical microscope (Zeiss). Root colonization, calculated as the percentage of fungus infection on the host plant, was determined by the slide method (Giovannetti and Mosse 1980) at 45, 70, 90 and 180 days of development.

The following parameters for Myc^+ and Myc^- in vitro clones and control mother plants at the vegetative stage (6 months old) were determined: mycorrhizal colonization (%); fresh and dry shoot weight; leaf area index; fresh and dry root weight; root/shoot fresh weight ratio. At the flowering stage (12 months old), the number of spicasters (inflorescence bearing stems), verticillasters (nodes of the inflorescence axis bearing two large branches of spikes), spikes (ovoid, compact) and florets (single flowers arranged in spikes) were determined.

Cytology

Root tips were treated for 4 h with a 0.05% aqueous solution of colchicine (Sigma) and then fixed in ethanol–acetic acid 3:1 (v/v). Standard Feulgen-staining and squashing procedures were used to obtain the preparations for the cytological analysis (Blanco et al. 1996).

Histology

The type of secretory glands and the density (number of glands mm^{-2}) of peltate trichomes on leaves from

plants of *O. vulgare L.* ssp. *hirtum* collected at different developmental stages were investigated following the procedure described by Avato et al. (2005).

Statistical analysis

Data were statistically processed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) multiple comparisons test with a significance level of $P \leq 0.05$.

Isolation and analysis of the essential oils

The aerial parts (2–60 g) of samples of *O. vulgare* ssp. *hirtum* were steam-distilled for 2.5 h with a Spring type apparatus (Albrigi, Italy) to recover the essential oils. The oil distillates were extracted from water with Et_2O, dried overnight over anhydrous Na_2SO_4, then kept in the refrigerator until analyzed.

Essential oils were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) as previously described (Avato et al. 2005). The identification of oil constituents was based on a comparison of their GC retention times combined with retention indices as well as by means of reference mass spectra from authentic standards and/or from library files (Avato et al. 2005).

Results

Micropropagation

The multiplication and growth of *O. vulgare* ssp. *hirtum* axillary buds was found to vary with the BAP concentration (Table 1). In agreement with that reported for *O. vulgare* ssp. *vulgare* by Ueno and Shetty (1998) and for *O. bastetanum* by Socorro et al. (1998), concentrations of 0.5 and 1 mg l^{-1} BAP gave the best explant responses (Table 1) in terms of percentage of growth (74–80%), number of developing shoots (3.0–3.2), shoot lenght (7.5–7.6 cm) and number of microcuttings (7.0–7.4). At the end of the subculture cycle, almost all of the plantlets had rooted, therefore auxin supplementation was not necessary; micropropagated plants had a 95% survival rate and acclimatized plants appeared normal during all of their physiological phases, up to and including the flowering stage. They did not show any morphological abnormalities or variations. Caryotyping confirmed that micropropagated oregano had a chromosome number of $2n = 30$, the same as the mother plant (Table 2).

Micropropagated clones were studied in detail at the flowering stage with respect to their morphological characters and essential oil production, which reaches the maximum at this phenological phase. Their homogeneity to the mother plants could be clearly seen (Table 2). As previously described in Morone-Fortunato et al. 2006, histological investigations of regenerated plantlets of *O. vulgare* at different developmental stages indicated that two types of secreting trichomes, capitate and peltate glandular hairs, were present on their leaf surface. Trichome morphology in the micropropagated plants of *O. vulgare* ssp. *hirtum* was found to be identical to that already described in literature for non-micropropagated plants (Bosabalidis 2002; Bosabalidis and Kokkini 1997; Morone-Fortunato and Circella 1993). A 12-celled sessile peltate hair with a peribasal crown of 18 radial epidermal cells from our micropropagated plants of *O. vulgare* ssp. *hirtum* is shown in Fig. 1a. Figure 1b shows the essential oil collected

Table 1 Effect of BAP concentrations on in vitro oregano explants after 4 weeks

BAP Concentration (mg l^{-1})	Necrosed explants (%)	Vitrificated explants (%)	Contaminated explants (%)	Grown explants (%)	*Shoots per explant (n)	*Length of shoots (cm)	*Microcuttings per shoot (n)
0.5	22	–	8	70	3.00 ± 0.35	7.50 ± 0.60	7.05 ± 0.25
1	10	–	10	80	3.19 ± 0.21	7.61± 0.57	7.45 ± 0.25
1.5	10	30	20	40	2.05 ± 0.21	6.02 ± 1.52	6.90 ± 0.45
2	20	55	25	–	–	–	–
Control	10	80	10	–	–	–	–

*Values are the means of 80 repetitions ± SE; each explant was a repetition. –, No response

Table 2 Chromosome number, peltate hair density, essential oil and carvacrol content of in vitro regenerated (VFB) and mother (MP) plants at the flowering stage

Parameters	VFB	MP
Chromosome number (2n)	30	30
*Adaxial leaf glands (n mm^{-2})	5.3 ± 0.1	5.5 ± 0.4
*Abaxial leaf glands (n mm^{-2})	4.5 ± 0.3	4.4 ± 0.3
Essential oil content (% dw)	1.6	1.4
Carvacrol (%)	84	89

*Values are the average of 5 representative samples of oregano leaves randomly excised from different plants ± SE

inside the cuticular layer shared by the cells forming the secretory pluricellular head of the peltate hair. As reported in Table 2, also the number of peltate secreting structures was not influenced by the in vitro culture, thus confirming the similarity of VFB plant material to the MP control plants.

The amount of essential oil synthesized by the in vitro clones of *Origanum vulgare* ssp. *hirtum* at the flowering stage (Table 2) was similar to that of the mother plants (1.6 vs 1.4% dw), with carvacrol being the main constituent in both oils (84 vs 89%).

Influence of mycorrhization on biomass production

Glomus viscosum induced root colonization in the in vitro clones of oregano within 45 days following infection (Table 3). However, at this stage of development, the intensity of colonization was less than half that of the control mother plants. The percentage of colonization thereafter rapidly increased in the micropropagated clones reaching the same level as in the mother tissues after 70 days of treatment. At 180 days after the infection, the influence of *G. viscosum* on micropropagated clones was evaluated on the basis of variations in the plant's fresh and dry leaf weight, leaf area index, fresh and dry root weight, and root/shoot fresh weight (Table 4). Not only was the leaf area in Myc^+ VF oregano plants more than 4 times larger compared to non-inoculated Myc^- clones, but it was almost 9 and 34 times larger,

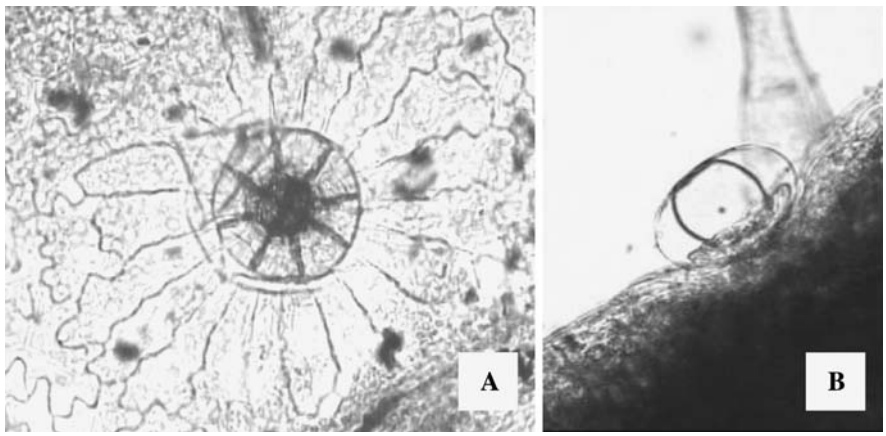

Fig. 1 Peltate glandular hairs of *Origanum vulgare* ssp. *hirtum* micropropagated plants: (**a**) paradermal section showing a 12-celled head, with a peribasal crown of 18 radial epidermal cells; (**b**) ortodermal section showing the essential oil secretion inside the cuticular layer of the gland head

Table 3 Mycorrhizal root colonization (%) of micropropagated plants and control mother plants from *O. vulgare* ssp. *hirtum* at four harvests

Plant samples	Days after infection#			
	45 days	70 days	90 days	180 days
Micropropagated plants	23.3 ± 2.7 b*	59.0 ± 3.7 a	68.0 ± 2.5 a	85.7 ± 3.7 a
Control mother plants	59.9 ± 5.6 a	67.0 ± 2.3 a	73.0 ± 2.1 a	85.4 ± 4.6 a

#Control non-mycorrhizal Myc^- plants did not show any infection

*Values are the means of 10 repetitions ± SE. Different letters indicate statistically ($P \leq 0.05$) significant differences between treatments (within a column) according to SNK's test

Table 4 Morphological parameters of non-mycorrhizal (Myc^-) and mycorrhizal (Myc^+) 6 months old *O. vulgare ssp. hirtum* (VF, micropropagated plants; MP6, control mother plants)

Parameters	VF		MP6	
	Myc^-	Myc^+	Myc^-	Myc^+
Leaf area (cm^2)	105.0 ± 1.98 b*	469.14 ± 20.53 a	13.83 ± 0.50 d	54.70 ± 0.50 c
Shoot number (n)	14.20 ± 0.36 b	31.20 ± 1.38 a	2.8 ± 0.20 d	6.0 ± 0.30 c
Shoot fresh weight (g)	8.40 ± 0.23 b	36.25 ± 1.94 a	0.49 ± 0.03 c	1.47 ± 0.06 c
Shoot dry weight (g)	1.86 ± 0.06 b	6.94 ± 0.36 a	0.13 ± 0.01 c	0.38 ± 0.006 c
Root fresh weight (g)	1.83 ± 0.04 b	7.15 ± 0.47 a	0.21 ± 0.002 c	0.47 ± 0.02 c
Root dry weight (g)	0.51 ± 0.01 b	2.35 ± 0.19 a	0.04 ± 0.004 c	0.08 ± 0.004 c
Root/shoot fresh weight	0.22 ± 0.002 c	0.19 ± 0.002 c	0.44 ± 0.45 a	0.32 ± 0.013 b

*Values are the means of 5 repetitions ± SE. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments (within a row) according to SNK's test

respectively, than that of the MP Myc^+ and Myc^- at the same growth stage. Fresh and dry weight of the aerial and underground organs also increased in Myc^+ plants and relatively more in the in vitro samples. On the other hand, the ratio between root and shoot fresh weight was always much lower than in the control material. The positive effect of AM was also evident at the flowering stage. The number of spicasters appeared significantly higher in Myc^+ VFB plant samples (Fig. 2).

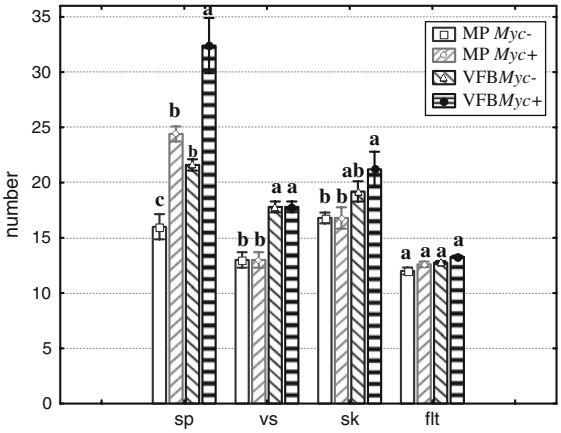

Fig. 2 The effect of mycorrhizal symbiosis on the number of spicasters/plant (sp), verticillasters/spicasters (vs), spikes/verticillaster (sk) and florets/spike (flt). Different letters indicate statistically significant ($P \leq 0.05$) differences for each parameter. Columns: values are the means of ten repetitions; vertical bars: ±SE; MP, 12 months old at flowering stage; VFB, micropropagated plants (12 months old) growing outdoors at the flowering stage; Myc^+, mycorrhizal and Myc^-, non-mycorrhizal

Performance of micropropagated *O. vulgare* ssp. *hirtum*: secretion glands and essential oils

Peltate hair density

The results from the analysis of variance (Table 5) showed the statistical significance of the mychorrizal effect and its interactions with the plant source, (MP6 and VF), on the total density of peltate hairs of 6-month-old plants. Table 6 reports the results from the SNK multiple test analysis of the only significant effects.

In agreement with previous reports on the species (Bosabilidis and Kokkini 1997; Werker et al. 1985), gland density was always higher on the upper leaf epidermis (Table 2). As shown in Fig. 3, the upper epidermis of Myc^+ micropropagated plants at 6 and 12 months had much more glands than the Myc^- micropropagated plants. Peltate hair density of Myc^+ micropropagated plants at 6 months was also higher compared to the control mother plants. This was not observed for 12-month-old Myc^+ micropropagated plants, probably due to the high number of spicasters, verticillasters and spikes at the flowering stage of growth (Fig. 2). The positive effect of AM on micropropagated plants was instead not significatively evident at 3 months.

During leaf development and expansion, the number of glandular hairs may remain stable or it may change (Bosabalidis 2002; Werker et al. 1985). An increase of the gland density following the physiological stages of growth of *O. vulgare* ssp. *hirtum* (Fig. 3) was observed for both micropropagated and

Table 5 Analysis of variance of plant and treatment effects and their interaction on peltate hair density values

Source	DF[a]	Peltate hair density#		P
		Mean square	F	
Plant[b]	1	0.45	0.88	0.3608 NS
Treatment[c]	1	5.618	11.04	0.0043[b]
Plant × Treatment	1	2.888	0.254	0.299[a]
Error	16	0.5085		
Total	19			

[a] DF: Degrees of freedom; [b]Plant: mother plants, MP6, and micropropagated plants, VF 6 months old; [c]Treatment: mycorrhizal, Myc^+ and non-mycorrhizal, Myc^-; #Peltate hair density, total gland number mm^{-2} of the upper and lower surface

Table 6 Means of peltate hair density relative to interaction (Plant × Treatment) and Treatment (mycorrhizal, Myc^+, non-mycorrhizal, Myc^-)

Plant	Treatment	
	Myc^+	Myc^-
MP6	7.62 a*	7.32 a*
VF	8.14 a*	6.26 b*
Means	7.88 a**	6.79 b**

*Interaction: Values with different letters are significantly different ($P \leq 0.05$); **Treatment: mycorrhizal, Myc^+ and non-mycorrhizal, Myc^-. Values with different letters are significantly different ($P \leq 0.05$); MP6, control mother plants, VF, micropropagated plants

control mother plants. Moreover, AM infection had little influence on the first 3 months of growth of the in vitro clones which had almost the same number of glands as the uninfected material suggesting a rejuvenation of the germplasm of those samples (Avato et al. 2005). Finally, the overall effect of the inoculation on the micropropagated plants can be seen as evidence of the excellent physiological conditions of the in vitro material as shown from the analysis of its growth parameters (Table 4).

Composition of the essential oils

Major constituents from GC and GC/MS analyses are reported in Table 7. Micropropagated clones had a chemical profile similar with that of the related control mother plants. Furthermore, the metabolite composition did not show any significant change due

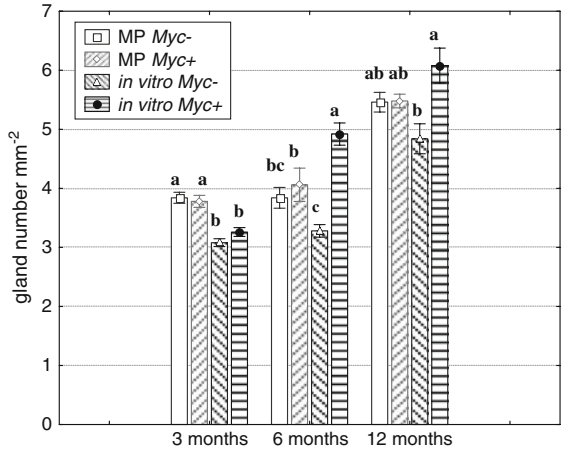

Fig. 3 Peltate hair density on upper leaf surface (gland number mm^{-2}). MP Myc^-, control mother plants; MP Myc^+, mycorrhizal control mother plants; in vitro Myc^-, micropropagated plants; in vitro Myc^+, mycorrhizal micropropagated plants Different letters indicate statistically significant ($P \leq 0.05$) differences for each harvest (3, 6 and 12 months). Columns: values are the means of ten repetitions; vertical bars: ±SE

to mycorrhizal inoculation. Carvacrol was the main component, its amount ranging from 81–95% of the essential oil. The rest was made up of other minor constituents, some present in all the samples, although at a different level: 1-octen-3-ol (0.1–0.5%), *p*-cymene (0.4–3.0%), γ-terpinene (tr-4.0%), *cis*- and *trans*-sabinene hydrate (0.1–0.3%), linalool (0.1–0.3%), borneol (0.06–1.0%), carvacrol methyl ether (0.1–1.6%), β-bisabolene (0.9–3.0%). Few variations were observed in the chemical composition of the volatile oils with respect to the different physiological stages of growth. However, when considering the overall composition, the essential oils from in vitro plants at a younger stage of growth, VA and VF, generally had a narrow range of constituents resembling the mother controls, MP3 and MP6. Instead, a larger variety of volatiles was observed in the 1-year-old inoculated and non-inoculated micropropagated flowering plants, VFB. In addition to carvacrol (84–81%, Myc^- and Myc^+ respectively), these clones synthesized more terpenoids as compared to the other plant clones. More specifically, total monoterpenoids amounted to 9.56 and 9.24% for Myc^- and Myc^+ respectively and total sesquiterpenoids to 3.32 and 3.98% for Myc^- and Myc^+ respectively. This compositional profile was also shared by the essential oils obtained from the MP

control plants (carvacrol, 89%; monoterpenoids, 8.11%; sesquiterpenoids, 2.93%). At the flowering stage of growth, a relatively higher amount of *p*-cymene and γ-terpinene was also observed (Table 7).

Discussion

The growth conditions adopted in this study allowed us to obtain micropropagated clones of *O. vulgare* ssp. *hirtum* which perfectly corresponded to the mother plant in terms of caryological and histological characteristics. Moreover, the micropropagated oregano described in this paper represented an excellent source of essential oils.

It is well-known that mycorrhization enhances biomass production in symbiotic plant partners also influencing the root/shoot ratio (Smith and Read 1997; Morone-Fortunato et al. 2005; Copetta et al. 2006). The positive effect of AM-related root colonization on the increase of the shoot biomass of oregano plants grown in a greenhouse has recently been reported by Khaosaad et al. (2006). The amount of shoot biomass in *O. vulgare* ssp. *hirtum* var. "Kalitera" was approximately three times larger than that of the control. However, as far as we know, data on the effects of AM fungi on micropropagated oregano plants are not available. The results of our study indicated that AM inoculation during the acclimatization phase and in the outdoor growth conditions induced positive effects on micropropagated clones of *O. vulgare* ssp. *hirtum.* The adaptation to mist greenhouse conditions was markedly facilitated by the presence of the mycorrhizal fungus. Six-month-old plants of Myc^+ *O. vulgare* ssp. *hirtum* showed better growth parameters than the control, confirming the improvement of AM colonization on the micropropagated plants. Root colonization was slower during the first 45 days of inoculation, becoming as efficient as in the control

Table 7 Major constituents (%) of the essential oils from *O. vulgare* ssp. *hirtum*

Compounds	*KI	[a]MP3	VA		MP6	VF		MP	VFB	
			Myc^-	Myc^+		Myc^-	Myc^+		Myc^-	Myc^+
α-Terpinene	1020	tr	tr	tr	tr	tr	tr	0.27	0.52	0.50
p-Cymene	1028	0.95	0.84	1.30	0.83	0.38	0.58	3.16	2.15	2.24
o-Cymene	1034	tr	–	–	–	tr	tr	–	0.19	0.23
trans-Ocimene	1054	–	tr	–	–	tr	tr	tr	tr	0.04
γ-Terpinene	1068	0.49	tr	0.06	0.29	0.11	0.13	1.64	3.99	3.71
1-Octen-3-ol	972	0.31	0.22	0.50	1.06	0.18	0.26	0.18	0.04	0.10
cis-Sabinene hydrate	1076	0.32	0.09	0.30	0.15	0.09	0.09	0.21	0.04	0.02
trans-Sabinene hydrate	1104	0.17	0.22	0.33	0.05	0.07	0.14	0.15	0.11	0.16
Linalool	1110	0.18	0.22	0.33	0.06	0.08	0.13	0.15	0.11	0.09
Borneol	1180	0.34	0.08	1.20	0.30	0.16	0.34	0.20	0.13	0.06
Terpinen-4-ol	1186	0.26	0.22	tr	0.17	0.11	0.22	0.30	tr	tr
α-Terpineol	1192	0.42	0.05	tr	0.07	0.13	0.31	0.11	tr	tr
cis-Dihydrocarvone	1200	–	0.07	tr	tr	0.11	0.09	0.17	tr	tr
Carvacrol methyl ether	1250	0.95	0.13	0.45	0.34	1.59	1.47.	0.10	0.13	0.15
Thymol	1284	tr	0.06	0.26	0.48	0.17	0.25	tr	tr	tr
Carvacrol	1301	93.88	94.27	89.02	89.68	90.75	89.25	88.72	83.72	80.67
Thymol acetate	1378	–	–	–	–	1.90	0.90	–	–	–
β-Caryophyllene	1416	0.45	0.51	0.65	0.56	0.96	0.16	0.98	1.13	1.43
β-Bisabolene	1516	0.90	1.48	3.10	1.57	1.25	1.81	1.70	1.75	1.92

*Kováts indexes (KI), relative to *n*-alkane series calculated on a DB-5 column

[a] MP, control mother plant (MP3, 3 months old; MP6, 6 months old; MP, 12 months old at flowering stage); VA, micropropagated plants (3 months old) during acclimatization; VF, micropropagated plants (6 months old) growing outdoor; VFB, micropropagated plants (12 months old) growing outdoors at the flowering stage; Myc^+, mycorrhizal and Myc^-, non-mycorrhizal

mother plants in the subsequent periods of growth (Table 3). Moreover, mycorrhization significantly affected all development parameters (Table 4), the number of peltate glands (Fig. 3) and the number of spicasters per plants and spikes per verticillasters (Fig. 2), suggesting that the performance of the in vitro plants was particularly improved by the combination of the two technological approaches, micropropagation and mycorrhization. The relatively lower ratio between root and shoot fresh weight in the Myc^+ plants was consistent with previous work (Berta et al. 2002; Copetta et al. 2006; Khaosaad et al. 2006; Smith and Read 1997). In conclusion, the reported physiological data indicate that even if the rate of colonization is the same it does not necessarily imply similar plant growth rate (Giovannetti 2006). In our study, micropropagated plants of *O. vulgare* ssp. *hirtum* proved to be more developed than the control plants even if they had the same level of infection (85% at 6 months; Tables 3 and 4). At the same level of infection, Myc^+ VF plants showed in fact better morphological parameters than Myc^- VF and Myc^+ and Myc^- MP control plants (Table 4), indicating that disease-free micropropagated plants of oregano, with no fungal or bacterial contamination, were a particularly good substrate for AM colonization and the absence of antagonists enhanced the rate of symbiosis.

As a consequence of the high number of secreting structures (average 8–11 and 6–10 in the upper and lower leaf side, respectively), *O. vulgare* ssp. *hirtum* is recognized as a plant with a high content of essential oil, even though its total yield is reported to be very variable (1–8% dw) (Bosabalidis 2002; Bosabalidis and Kokkini 1997; Skoula and Harborne 2002). In comparison to the above, peltate hairs on our oregano MP clone had a lower density (5.5 ± 0.4 and 4.4 ± 0.3, upper and lower epidermis respectively; Table 2) and, as expected, an intermediate yield of oil is produced (1.4% dw; Table 2). The histological analysis clearly showed that density of sessile glands reflects the physiological ageing of the plant tissue (Fig. 3), reaching and exceeding in Myc^+ VFB and Myc^+ VF, respectively, the density of the MP sample. Consistently with these morphological characteristics, the production of the essential oil in the micropropagated phase Myc^+ VFB, proved to be higher than in the MP control, thus demonstrating that our micropropagated clones of *O. vulgare* ssp. *hirtum* are quantitatively a very good source of useful secondary metabolites (Table 7).

Carvacrol was the main volatile in the *O. vulgare* ssp. *hirtum* used in this investigation. Compared to the control plants, the content of this metabolite proved to be stable and was also replicated in the in vitro plants (Table 7), independently of the age of the tissues. The slight decrease in its content observed during the plant development was compensated for by the increase of minor monoterpenoid and sesquiterpenoid constituents. Previous investigations (Johnson et al. 2004) have shown that also γ-terpinene and *p*-cymene are typical volatiles in this plant. These three metabolites are biosynthetically related; the synthesis of carvacrol proceeds from γ-terpinene through to *p*-cymene (Dewick 2001). Environmental and ontogenetic variations on the relative content of these metabolites in *O. vulgare* ssp. *hirtum* have been reported (Johnson et al. 2004) and carvacrol was found to become dominant over *p*-cymene during the plant development. γ-Terpinene was also found to decrease from younger to older leaves.

Our results indicate that enzymic conversion of *p*-cymene to carvacrol is very efficient in oregano plants before they reach the flowering stage. What is more interesting is that this behavior is shared by cuttings as well as by micropropagated plants (Table 7). On flowering, the biosynthetic capacity of the regenerated plants of oregano, as in the mother plants, becomes less specific and some of the other intermediates are released in the pathway to carvacrol. A similar study on micropropagation of *Salvia officinalis* (Avato et al. 2005) displayed a chemical profile which changed with the age of the plant and could be related to the activation of different routes in the overall terpenoid metabolism.

The chemical profile of *O. vulgare* ssp. *hirtum* was little influenced by the fungus symbiosis (Table 7). Some studies have been 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 amni* (Kapoor et al. 2002b). Experiments on *Ocimum basilicum* (Copetta et al. 2006) led to the conclusion that different fungi can modulate the yield of essential oils differently in the same plant. Thus, mycorrhization of this species with *G. mosseae* did not affect the production of volatile oils, in contrast to *Gigaspora margarita* and *Gi. rosea*. Similarly, inoculation of *O. vulgare* ssp. *hirtum* with *G. mosseae* did not alter the concentration of the essential oils (Khaosaad et al. 2006). As shown in Table 4 and Fig. 3, mycorrhization with *G. viscosum* of our micropropagated oregano greatly improved the development of 6-month-old plants (VF), producing significant increases in their gland density on the upper epidermis, leaf area and fresh and dry weight. An increase of the verticillaster and spicaster numbers of the micropropagated clones of *O. vulgare* ssp. *hirtum* was also observed up to the flowering phase of growth (Fig. 2). However, this was probably not enough to produce clear changes in the synthesis and accumulation of essential oils, thus explaining the relatively uniform chemical profile between micropropagated plants (Myc^+ and Myc^-) and oregano control plants (Table 7).

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