

# Glandular trichomes and essential oil characteristics of *in vitro* propagated *Micromeria pulegium* (Rochel) Benth. (Lamiaceae)

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## Abstract

**Main conclusion** *In vitro* conditions and benzyladenine influenced both content and composition of micropropagated *Micromeria pulegium* essential oils, with pulegone and menthone being the main essential oil components.

The content and chemical composition of *Micromeria pulegium* (Rochel) Benth. essential oils were studied in native plant material at vegetative stage and in micropropagated plants, obtained from nodal segments cultured on solid MS medium supplemented with N<sup>6</sup>-benzyladenine (BA) or kinetin at different concentrations, alone or in combination with indole-3-acetic acid. Shoot proliferation was achieved in all treatments, but the highest biomass production was obtained after treatment with 10 μM BA. Phytochemical analysis identified up to 21 compounds in the essential oils of wild-growing and *in vitro* cultivated plants, both showing very high percentages of total

monoterpenoids dominated by oxygenated monoterpenes of the menthane type. Pulegone and menthone were the main essential oil components detected in both wild-growing plants (60.07 and 26.85 %, respectively) and micropropagated plants grown on either plant growth regulator-free medium (44.57 and 29.14 %, respectively) or BA-supplemented medium (50.77 and 14.45 %, respectively). The percentage of total sesquiterpenoids increased *in vitro*, particularly owing to sesquiterpene hydrocarbons that were not found in wild-growing plants. Differences in both content and the composition of the essential oils obtained from different samples indicated that *in vitro* culture conditions and plant growth regulators significantly influence the essential oils properties. In addition, the morphology and structure of *M. pulegium* glandular trichomes in relation to the secretory process were characterized for the first time using SEM and light microscopy, and their secretion was histochemically analyzed.

**Keyword** Axillary bud induction · Capitate trichomes · Histochemistry · Micropropagation · Peltate trichomes · Phytochemical analysis

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## Abbreviations

BA	N <sup>6</sup> -benzyladenine
GC–MS	Gas chromatography–mass spectrometry
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog’s medium
NAA	1-naphthaleneacetic acid
PGR	Plant growth regulator
SEM	Scanning electron microscopy

## Introduction

The genus *Micromeria* Benth. (Lamiaceae) comprises more than 70 herbs, subshrubs and shrubs distributed from the Himalayan region to the Macaronesia and from the Mediterranean to South Africa and Madagascar (Harley et al. 2004). Based on morphological characteristics and phylogenetic relationships, species from the genus *Micromeria* are grouped into four sections: *Pseudomelissa* Benth., *Micromeria*, *Cymularia* Boiss. and *Pineolentia* P. Pérez (Harley et al. 2004). Section *Pseudomelissa* is one of the most complex taxa, with still unstable systematic position within *Mentheae* tribe of *Nepetoideae* subfamily. Based on molecular evidence, emphasizing the similarity of selected morphological traits, Bräuchler et al. (2006) currently include species of *Micromeria*, sect. *Pseudomelissa* in genus *Clinopodium* L.

In the past years, several reports have been published on the chemotaxonomical traits of *Micromeria* species showing the presence of biologically active phenolic compounds and the essential oils (Vladimir-Knežević et al. 2011; Kremer et al. 2012, 2014). Species belonging to the genus *Micromeria* contain considerable quantities (>0.5 %) of essential oils dominated by oxygenated monoterpenes of the menthane class (Slavkovska et al. 2005), which exhibit substantial antimicrobial (Duru et al. 2004; Šavikin et al. 2010) and antioxidant activities (Vladimir-Knežević et al. 2011; Tošić et al. 2015), thus protecting the plant from pathogen attacks. The essential oil composition within the genus *Micromeria* displays great variability among species, with significant seasonal variations (Slavkovska et al. 2005, 2013).

*Micromeria pulegium* (Roche) Benth. is a perennial, medium-sized, erected plant. Green parts of the plant (especially leaves) are densely covered by small glands, rendering the plant pleasant aroma. This species inhabits screes and sheltered calcareous rocks, mainly in the gorges, at altitudes of 1000–1200 m. *M. pulegium* is an endangered taxon endemic to South Carpathians with scattered native area in Romania and with one single known locality in Eastern Serbia, and therefore protected by law in category of protected species in Serbia (Serbian Ministry of Environment and Physical Planning and Ministry of Agriculture, Forestry and Water Management 2010, 2011). The yield and the composition of the essential oils of *M. pulegium* recorded from natural populations within the territory of Eastern Serbia and from cultivated plants through different phenological stages were reported by Slavkovska et al. (2013). Both natural and cultivated populations were characterized by high amounts of the essential oils (0.8–1.4 %) at all stages of development. Chemical composition and antioxidant and antimicrobial

activities of extracts of native and in vitro micropropagated *M. pulegium* plants have been recently reported by Tošić et al. (2015).

Because the chemical synthesis of most pharmaceutically important secondary metabolites is not economically convenient, these compounds are isolated from wild-growing or cultivated plants (Oksman-Caldentey and Inzé 2004). However, a number of publications reported on the shortage in natural resources of many medicinal and aromatic plants all over the world (Hamilton 2004). An increasing demand for plant-derived medicines confirms the importance of the establishment of an efficient plant tissue culture system with high-frequency plant regeneration for the production of pharmacologically active compounds. In addition, in vitro culture techniques can help overcome the individual variability, due to genetic and biochemical heterogeneity, which is the major difficulty in the use of the Lamiaceae species in the pharmaceutical industry (Saha et al. 2012).

Micropropagation is a valuable method for the multiplication of selected genotypes and chemotypes, leading to high regeneration rates of many medicinal and aromatic plants in vitro (Rout et al. 2000). These plants can be subsequently used for a variety of studies, thus avoiding collection from their natural habitat. In vitro propagation from wild-growing plants through axillary shoot formation reduces the possibility of the occurrence of abnormalities that exist with other methods (Kalemba and Thiem 2004). Along with somatic embryogenesis and adventitious shoot induction, micropropagation can efficiently serve to propagate clonally plants under controlled conditions, regardless of the season and to induce quantitative and qualitative modifications in the production of plant secondary metabolites by changing nutrient and hormonal composition of the growth medium, or different physical factors (Collin 2001). Several authors have shown that under certain culture conditions, the essential oil production could be increased without changing its composition (Santos-Gomes and Fernandes-Ferreira 2003; Affonso et al. 2009).

Wild-growing plants of *M. pulegium* are characterized by relatively high amounts of the essential oils rich in pulegone with a potential use as a bio-insecticide and a bio-pesticide (Koul et al. 2008). In view of the potential pharmacological and commercial value of this species, the present study was initiated to phytochemically examine the influence of in vitro culture on the production of the essential oils in micropropagated plants of *M. pulegium*. The effect of plant growth regulators on *M. pulegium* propagation through axillary bud induction was investigated, and the morphology and structure of *M. pulegium* glandular hairs in relation to the secretory process were

characterized. In addition to assuring uniformity of plant material for the characterization of the essential oils, *in vitro* propagated plants can be subsequently used for *ex situ* conservation of this endemic and rare species.

## Materials and methods

### Plant material

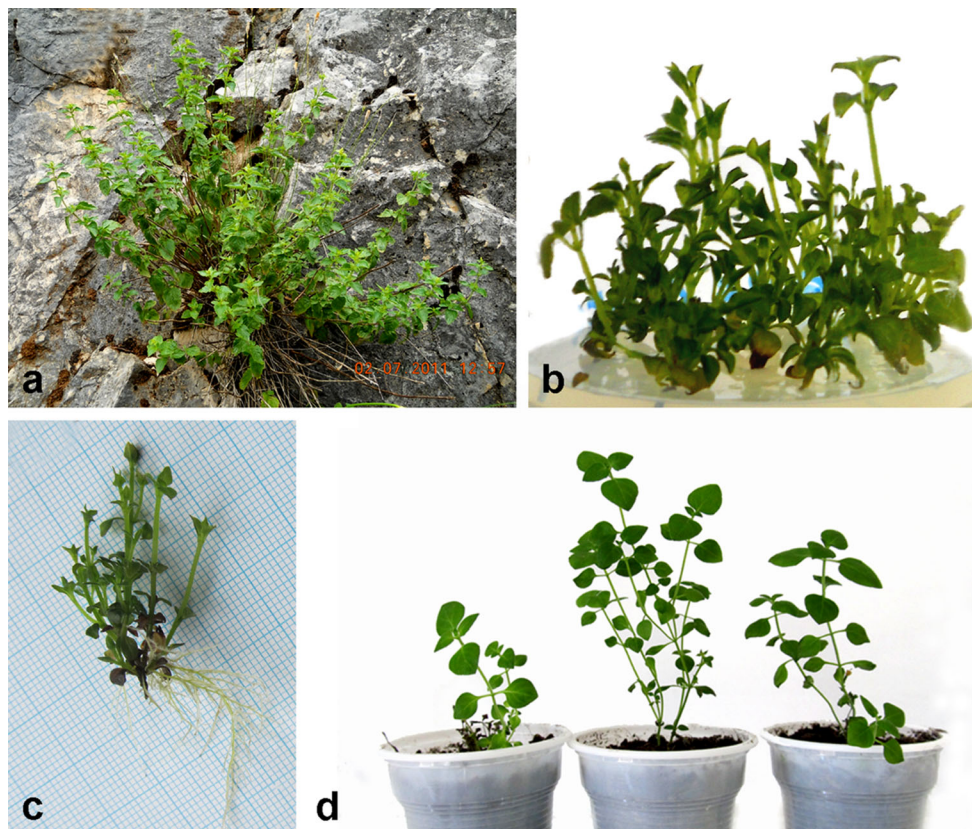
Aerial parts (shoots) of *M. pulegium* plants at the vegetative stage of development were randomly collected from wild-growing (natural) populations in Svrlijski Timok gorge, at latitude 43°32'23"N and longitude 22°10'16"E in August 2012 (Fig. 1a). Voucher specimen (N° 6912) was deposited in the Herbarium collection of the Faculty of Science and Mathematics, University of Niš (HMN).

### Micropropagation

Shoots dissected into one-node stem segments (~1 cm) bearing two axillary buds were used as explants to establish *in vitro* cultures, as described by Tošić et al. (2015). Nodal

segments were surface-sterilized with 25 % commercial sodium hypochlorite solution (6 % active chlorine) containing two drops of liquid commercial detergent for 30 min and rinsed three times with sterile distilled water. Afterward, explants were placed in the solution containing 500 mg L<sup>-1</sup> nystatin for 24 h and rinsed three times with sterile distilled water. Micropropagation was carried out on basal MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose (w/v) and 0.7 % (w/v) agar (Torlak, Belgrade) in 250-mL glass jars containing 25 mL of the medium. Ten explants were placed in each jar closed with polycarbonate cover. The pH of the media was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min. Cultures were maintained in a growth chamber under a 16-h photoperiod, with a photon flux density 45 μE m<sup>-2</sup> s<sup>-1</sup> provided by cool white fluorescent lamps, at 25 ± 2 °C. Routine subculture was performed in 4-week intervals (Tošić et al. 2015).

Nodal segments derived from shoot cultures grown on basal medium for four weeks were transferred to MS medium supplemented with various plant growth regulators (PGRs) at different concentrations: N<sup>6</sup>-benzyladenine (BA) at 0.1, 0.3, 1, 3, 10 or 30 μM; kinetin (Kin) at 0.1,



**Fig. 1** Micropropagation of *Micromeria pulegium*. **a** Wild-growing plant. **b** *In vitro* plantlets cultured on PGR-free medium, used for trichome characterization. **c** Rooting on culture medium

supplemented with 5.71 μM IAA. **d** Acclimatized *M. pulegium* plantlets regenerated via axillary shoots

0.3, 1, 3, 10 or 30  $\mu\text{M}$ ; 0.57  $\mu\text{M}$  indole-3-acetic acid (IAA); 0.1–30  $\mu\text{M}$  BA in combination with 0.57  $\mu\text{M}$  IAA; and 0.1–30  $\mu\text{M}$  Kin in combination with 0.57  $\mu\text{M}$  IAA. Basal MS medium devoid of PGRs was used as a control. After 4 weeks in culture, the number of explants producing shoots, the number of shoots per explant and the shoot length, as well as explant fresh and dry weight, were recorded in order to evaluate the effect of PGRs on shoot multiplication.

For root induction, individual axillary shoots (15–20 mm) were harvested after the multiplication stage from explants grown on PGR-free MS medium and transferred to half-strength MS medium lacking growth regulators or supplemented with IAA, indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA) at following concentrations: 0.57, 1.14, 1.71 and 5.71  $\mu\text{M}$  for IAA; 0.49, 0.98, 1.48 and 4.9  $\mu\text{M}$  for IBA; 0.54, 1.07, 1.61 and 5.37  $\mu\text{M}$  for NAA. The percentage of rooted shoots and the root length were recorded after 5 weeks in culture. Well-rooted shoots were removed from the medium, washed with water to remove any adherent culture medium and transferred to plastic pots with soil mixture (60 % peat, 20 % red worm compost and 20 % sand), enclosed in a thin transparent plastic envelope to reduce water loss and maintained in the greenhouse. Plantlets were gradually acclimatized over a 4-week period.

### Scanning electron microscopy

For scanning electron microscopy (SEM), fresh leaves isolated from shoots cultured on MS without PGRs were used. Leaf samples were coated with a thin layer of gold and palladium in a BAL-TEC SCD 005 sputter coater. Both adaxial and abaxial surfaces were examined with a JEOL JSM-6390 LV (JEOL, Tokyo, Japan) scanning electron microscope operated at 15 kV.

### Light microscopy

Leaf specimens were fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C. Following a 2-h wash in the same buffer, material was postfixed in 1 % osmium tetroxide in phosphate buffer at 4 °C overnight. Fixed material was washed, dehydrated and embedded in Araldite resin CY 212 (Agar Scientific Ltd. England), as described by Glauert and Glauert (1958). Semithin cross sections (1–1.5  $\mu\text{m}$  thick) were cut on LKB III ultramicrotome, stained with methylene blue and examined under a Zeiss Axiovert light microscope (Carl Zeiss GmbH, Göttingen, Germany).

### Histochemical characterization

Histochemical analyses were performed on hand-sections of fresh leaves, in order to detect the presence of lipophilic substances in the actively secreting trichomes. The following histochemical tests were used: Sudan Black B and Sudan IV for total lipids (Jensen 1962); Nile Blue A for neutral and acidic lipids (Cain 1947); and Nadi reagent for terpenes (David and Carde 1964). For all the histochemical methods used, control tests were carried out following the suggestions of the respective authors. Sections were examined and photographed using a Zeiss Axiovert light microscope (Carl Zeiss GmbH, Göttingen, Germany).

### Isolation and analysis of the essential oils

Wild-growing and micropropagated plants at the vegetative stage of development were used for the essential oil analysis. The essential oils from the aerial parts (shoots) of the plants dried at room temperature were obtained by hydrodistillation for 3 h in a Clevenger-type apparatus according to the procedure given in The European Pharmacopoeia (2011). The essential oils were dissolved using *n*-hexane, dried over anhydrous sodium sulfate and kept at –40 °C until analysis. The oil yield was expressed in mL per 100 g of dry weight of the plant material.

Volatile constituents were determined by GC and GC–MS analyses. GC and GC–MS analyses were performed on an Agilent 6890 N GC system equipped with 5975 MSD and FID, using a HP-5 MS column (30 m  $\times$  0.32 mm  $\times$  0.25  $\mu\text{m}$ ). Injection volume was 1  $\mu\text{L}$  and injector temperature was 200 °C with a 10:1 split ratio. Helium was a carrier gas and its flow rate was 1.0 mL  $\text{min}^{-1}$  (constant flow mode). Column temperature was linearly programmed in the range from 60 to 280 °C at a rate of 3 °C  $\text{min}^{-1}$  and held at 280 °C for 5 min. The transfer line was heated at 250 °C. The FID detector temperature was 300 °C. EI mass spectra (70 eV) were acquired in the *m/z* range 35–550. The retention indices were experimentally determined using *n*-alkanes (C8–C20 and C21–C40) injected after the essential oil, under the same chromatographic conditions.

The identification of the compounds was based on the comparison of their retention indices (RI), their retention times and mass spectra with those obtained from authentic samples and/or the NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, Wiley libraries, Adams database and literature (Adams 2001). Relative percentages of the identified compounds were computed from the GC–FID peak area.

**Statistical analysis**

The efficiency of different PGR treatments on growth rate was determined by comparing biomass increase and in vitro proliferation rates, using different methods. Biomass increase was calculated on both fresh and dry weight basis. Proliferation rate was assessed by counting the number of shoots at subculture and following 4-week PGR treatment under previously defined conditions. The data were obtained from two repeated experiments, with sixty explants per each medium (ten explants/jar). Percentage data were subjected to angular transformation prior to analysis. The data were averaged and statistically analyzed, and differences were tested for significance using ANOVA multiple range test at the significance level of  $P \leq 0.05$ .

**Results and discussion**

**Shoot multiplication**

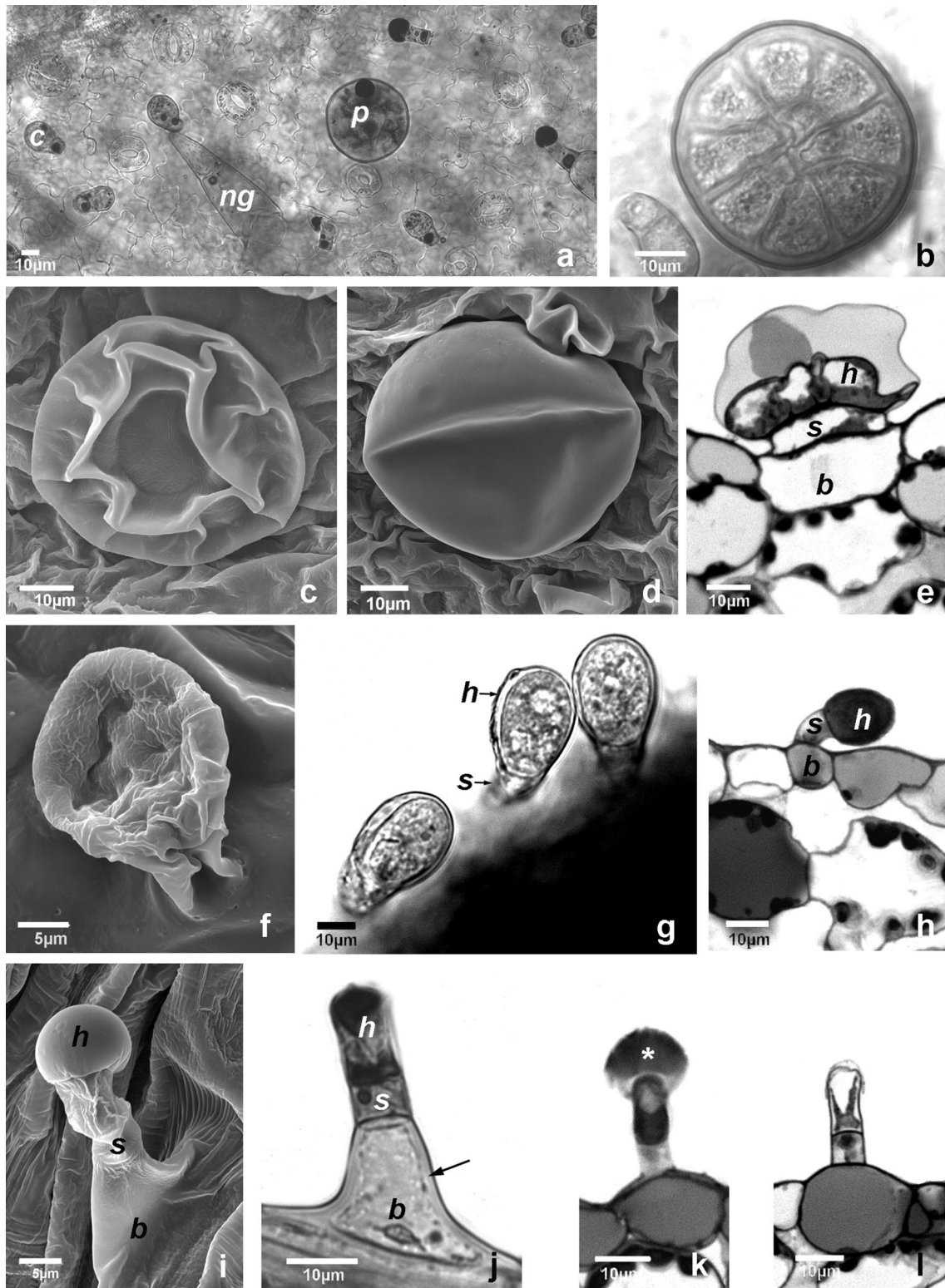
The nodal explants of *M. pulegium* cultured on MS medium without plant growth regulators produced shoots (Fig. 1b). This contradicts the results obtained for different species of Lamiaceae family grown in vitro, where shoot proliferation required application of cytokinins (usually Kin or BA), alone or with auxin at low concentration (Saha et al. 2012; Bakhtiar et al. 2014). On media supplemented with cytokinins, the percentage of explants developing shoots was high for both BA and kinetin (Table 1).

Maximum axillary bud proliferation was obtained at 3  $\mu$ M BA, whereas Kin did not increase shoot proliferation (Table 1). However, shoots cultured on medium supple-

**Table 1** Effect of plant growth regulators on *Micromeria pulegium* shoot proliferation, number of shoots per explant, shoot length and shoot fresh and dry weight after 28 days of culture

PGR ( $\mu$ M)			Explants producing shoots (%)	Number of shoots per explant	Shoot length (mm)	Explant fresh weight (g)	Explant dry weight (g)
BA	Kin	IAA					
–	–	–	100.0 $\pm$ 0.0 <sup>h</sup>	15.90 $\pm$ 1.05 <sup>defgh</sup>	8.56 $\pm$ 0.29 <sup>bcdefg</sup>	0.17 $\pm$ 0.01 <sup>cdef</sup>	0.02 $\pm$ 0.001 <sup>bcde</sup>
0.1	–	–	93.3 $\pm$ 0.1 <sup>fg</sup>	12.86 $\pm$ 0.79 <sup>ab</sup>	7.70 $\pm$ 0.28 <sup>ab</sup>	0.14 $\pm$ 0.008 <sup>abc</sup>	0.02 $\pm$ 0.008 <sup>ab</sup>
0.3	–	–	66.7 $\pm$ 0.1 <sup>ab</sup>	11.70 $\pm$ 1.29 <sup>a</sup>	8.67 $\pm$ 0.45 <sup>cdefgh</sup>	0.13 $\pm$ 0.01 <sup>ab</sup>	0.02 $\pm$ 0.01 <sup>ab</sup>
1	–	–	58.3 $\pm$ 0.1 <sup>a</sup>	16.43 $\pm$ 1.10 <sup>efgh</sup>	9.00 $\pm$ 0.48 <sup>defghi</sup>	0.21 $\pm$ 0.01 <sup>gh</sup>	0.02 $\pm$ 0.01 <sup>cdef</sup>
3	–	–	100.0 $\pm$ 0.0 <sup>h</sup>	19.42 $\pm$ 0.92 <sup>i</sup>	9.31 $\pm$ 0.30 <sup>ghij</sup>	0.23 $\pm$ 0.01 <sup>hi</sup>	0.02 $\pm$ 0.01 <sup>efg</sup>
10	–	–	100.0 $\pm$ 0.0 <sup>h</sup>	16.62 $\pm$ 0.83 <sup>fgh</sup>	9.75 $\pm$ 0.31 <sup>ijkl</sup>	0.31 $\pm$ 0.01 <sup>m</sup>	0.03 $\pm$ 0.01 <sup>j</sup>
30	–	–	100.0 $\pm$ 0.0 <sup>h</sup>	15.62 $\pm$ 0.80 <sup>cdefg</sup>	9.11 $\pm$ 0.22 <sup>fghij</sup>	0.26 $\pm$ 0.02 <sup>jk</sup>	0.03 $\pm$ 0.001 <sup>fgh</sup>
–	–	0.57	70.0 $\pm$ 0.1 <sup>ab</sup>	16.21 $\pm$ 1.40 <sup>efgh</sup>	8.15 $\pm$ 0.35 <sup>abcde</sup>	0.16 $\pm$ 0.01 <sup>bcde</sup>	0.02 $\pm$ 0.002 <sup>abcd</sup>
0.1	–	0.57	93.3 $\pm$ 0.1 <sup>fg</sup>	14.68 $\pm$ 1.42 <sup>abcdef</sup>	7.59 $\pm$ 0.32 <sup>a</sup>	0.14 $\pm$ 0.01 <sup>ab</sup>	0.02 $\pm$ 0.001 <sup>a</sup>
0.3	–	0.57	76.7 $\pm$ 0.1 <sup>bcde</sup>	12.87 $\pm$ 0.85 <sup>abc</sup>	10.58 $\pm$ 0.51 <sup>l</sup>	0.18 $\pm$ 0.01 <sup>defg</sup>	0.02 $\pm$ 0.002 <sup>cdef</sup>
1	–	0.57	96.7 $\pm$ 0.1 <sup>gh</sup>	14.66 $\pm$ 0.82 <sup>abcdef</sup>	9.07 $\pm$ 0.34 <sup>fghij</sup>	0.20 $\pm$ 0.007 <sup>fgh</sup>	0.02 $\pm$ 0.001 <sup>bcde</sup>
3	–	0.57	100.0 $\pm$ 0.0 <sup>h</sup>	17.33 $\pm$ 0.98 <sup>fghi</sup>	10.13 $\pm$ 0.32 <sup>kl</sup>	0.27 $\pm$ 0.01 <sup>jk</sup>	0.03 $\pm$ 0.001 <sup>ghij</sup>
10	–	0.57	100.0 $\pm$ 0.0 <sup>h</sup>	18.19 $\pm$ 1.11 <sup>ghi</sup>	9.94 $\pm$ 0.28 <sup>kl</sup>	0.30 $\pm$ 0.02 <sup>lm</sup>	0.03 $\pm$ 0.02 <sup>ij</sup>
30	–	0.57	100.0 $\pm$ 0.0 <sup>h</sup>	15.62 $\pm$ 0.89 <sup>cdefg</sup>	8.50 $\pm$ 0.23 <sup>bcdef</sup>	0.27 $\pm$ 0.02 <sup>jkl</sup>	0.03 $\pm$ 0.01 <sup>ghi</sup>
–	0.1	–	80.0 $\pm$ 0.1 <sup>bcde</sup>	14.52 $\pm$ 1.55 <sup>abcdef</sup>	7.94 $\pm$ 0.33 <sup>abc</sup>	0.16 $\pm$ 0.01 <sup>bcde</sup>	0.02 $\pm$ 0.002 <sup>abcd</sup>
–	0.3	–	75.0 $\pm$ 0.1 <sup>bcd</sup>	12.44 $\pm$ 1.27 <sup>ab</sup>	7.51 $\pm$ 0.32 <sup>a</sup>	0.12 $\pm$ 0.01 <sup>a</sup>	0.01 $\pm$ 0.001 <sup>a</sup>
–	1	–	80.0 $\pm$ 0.1 <sup>cde</sup>	18.75 $\pm$ 1.32 <sup>hi</sup>	8.06 $\pm$ 0.30 <sup>abcd</sup>	0.19 $\pm$ 0.01 <sup>defg</sup>	0.02 $\pm$ 0.001 <sup>def</sup>
–	3	–	68.3 $\pm$ 0.1 <sup>ab</sup>	13.22 $\pm$ 1.32 <sup>abcde</sup>	7.46 $\pm$ 0.34 <sup>a</sup>	0.13 $\pm$ 0.01 <sup>ab</sup>	0.02 $\pm$ 0.001 <sup>a</sup>
–	10	–	88.3 $\pm$ 0.2 <sup>fg</sup>	12.51 $\pm$ 0.94 <sup>ab</sup>	9.39 $\pm$ 0.39 <sup>ghijk</sup>	0.16 $\pm$ 0.01 <sup>bcd</sup>	0.02 $\pm$ 0.001 <sup>ab</sup>
–	30	–	85.0 $\pm$ 0.1 <sup>ef</sup>	16.87 $\pm$ 0.88 <sup>fghi</sup>	8.78 $\pm$ 0.27 <sup>defgh</sup>	0.23 $\pm$ 0.01 <sup>hi</sup>	0.02 $\pm$ 0.001 <sup>cdef</sup>
–	0.1	0.57	81.7 $\pm$ 0.1 <sup>de</sup>	14.80 $\pm$ 0.88 <sup>abcdef</sup>	8.68 $\pm$ 0.34 <sup>defgh</sup>	0.18 $\pm$ 0.01 <sup>defg</sup>	0.02 $\pm$ 0.001 <sup>def</sup>
–	0.3	0.57	93.3 $\pm$ 0.1 <sup>fg</sup>	16.07 $\pm$ 0.97 <sup>efgh</sup>	7.73 $\pm$ 0.29 <sup>ab</sup>	0.16 $\pm$ 0.01 <sup>bcde</sup>	0.02 $\pm$ 0.001 <sup>bcd</sup>
–	1	0.57	83.3 $\pm$ 0.1 <sup>cde</sup>	15.16 $\pm$ 0.89 <sup>bcdef</sup>	9.70 $\pm$ 0.39 <sup>hijkl</sup>	0.20 $\pm$ 0.01 <sup>fgh</sup>	0.02 $\pm$ 0.001 <sup>def</sup>
–	3	0.57	83.3 $\pm$ 0.1 <sup>de</sup>	13.14 $\pm$ 0.68 <sup>abcd</sup>	9.10 $\pm$ 0.35 <sup>fghij</sup>	0.19 $\pm$ 0.01 <sup>efg</sup>	0.02 $\pm$ 0.001 <sup>bcde</sup>
–	10	0.57	100.0 $\pm$ 0.0 <sup>h</sup>	18.0 $\pm$ 1.05 <sup>ghi</sup>	9.02 $\pm$ 0.27 <sup>efghi</sup>	0.28 $\pm$ 0.02 <sup>klm</sup>	0.03 $\pm$ 0.002 <sup>hij</sup>
–	30	0.57	100.0 $\pm$ 0.0 <sup>h</sup>	16.78 $\pm$ 0.85 <sup>fghi</sup>	9.06 $\pm$ 0.25 <sup>fghi</sup>	0.25 $\pm$ 0.01 <sup>ij</sup>	0.03 $\pm$ 0.004 <sup>ij</sup>

Values are mean  $\pm$  SE,  $n = 60$ . Means in the column followed by different letters are different according to LSD multiple range test ( $P \leq 0.05$ )



mented with Kin had somewhat healthier appearance since hyperhydricity frequently occurred on BA-supplemented media. One of the most important factors known to induce

vitrication is the excess of cytokinins in the culture medium (Leshem et al. 1988; Zuzarte et al. 2010). In combination with IAA neither of the applied cytokinins

**Fig. 2** SEM and light micrographs of in vitro leaf trichomes of *Micromeria pulegium*. **a** The abaxial surface of leaf lamina showing both non-glandular (*ng*) and two types of glandular trichomes, peltate (*p*) and capitate (*c*), stained with NADI reagent. **b** Upper view of glandular head of fully developed peltate trichome, with eight secretory cells arranged in a disk. **c** Glandular head of immature peltate trichome with the cuticular cap attached to the head cells. **d** Glandular head of peltate trichome with developing subcuticular space during maturation. **e** Longitudinal section of mature peltate trichome with broad vacuolated basal cell (*b*) and short unicellular stalk (*s*) subtending the multicellular head (*h*). Note osmiophilic secretion in the large subcuticular space. **f** Upper view of capitate trichome type I. **g** Capitate trichome type I, with a short unicellular stalk (*s*) subtending unicellular glandular head (*h*) stained with Sudan IV. **h** Longitudinal section of the secretory-stage capitate trichome type I, showing the basal cell (*b*), unicellular stalk (*s*) and unicellular head (*h*). **i** Capitate trichome type II with elongated basal cell (*b*), short stalk (*s*) and glandular head with round tip (*h*). **j** Capitate trichome type II stained with Sudan Black B, showing vacuolated pyramidal basal cell (*b*) with thick cell wall (*arrow*) subtending cylindrical unicellular stalk (*s*) and glandular head (*h*), both of approximately same diameter. **k** Longitudinal section of capitate trichome type II, showing more vacuolated stalk cell with large nucleus, and apical secretory cell rich in cytoplasm with large subcuticular space (*asterisk*) filled with osmiophilic secretion. **l** Longitudinal section of capitate trichome type II, following cuticle rupture and the release of accumulated secretory product

significantly affected axillary shoot induction (Table 1). However, IAA slightly increased shoot elongation (up to  $10.58 \pm 0.51$  mm) when applied with BA. In contrast, Santoro et al. (2013) showed that supplementation with BA alone resulted in the highest values for shoot and root length.

Compared to medium without PGRs, neither of the cytokinins at lower concentrations increased the biomass production. BA at higher concentrations (10–30  $\mu$ M BA) led to an increase in both fresh and dry weight per explant, regardless of the presence of auxin in the medium. Maximum biomass production was achieved on medium supplemented with 10  $\mu$ M BA (Table 1). This contradicts the findings of Santoro et al. (2013), who showed that supplementation with BA alone resulted in the lowest values for shoot fresh weight in *Mentha piperita*, whereas the highest shoot fresh weight was obtained on medium supplemented with auxin (IBA), alone or in combination with BA. The effect of Kin on both fresh and dry biomass production was strongest when applied at 10  $\mu$ M, in combination with 0.57  $\mu$ M IAA. In general, Kin was more effective in increasing the biomass production when applied in combination with auxin (Table 1).

### Rooting and acclimatization

Spontaneous rooting of micropropagated shoots was frequently observed in a number of Lamiaceae species such as *Lavandula pedunculata* (Zuzarte et al. 2010), *Salvia*

*dolomitica* (Bassolino et al. 2015) and others, where roots were induced on PGR-free medium, or *Origanum vulgare*, where rooting did not require auxin supplementation but occurred at the end of the subculture cycle on BA-supplemented medium (Morone-Fortunato and Avato 2008). Spontaneous rooting that avoids the use of auxins known to induce callus and anomalous vascular connections between shoots and adventitious roots is advantageous for in vitro propagation. However, in *M. pulegium* only about 28 % of the shoots rooted spontaneously on the PGR-free medium; hence, the auxin treatment was used to promote rooting. Of all the auxins tested, IAA was the most efficient in promoting root induction (54 % at 5.71  $\mu$ M IAA) as well as in subsequent root growth (Fig. 1c). Neither of the tested IBA concentrations stimulated rooting, whereas NAA induced few short roots only when applied at higher concentrations (data not shown).

Seventy-eight percent of the rooted plantlets were successfully acclimatized 4 weeks upon transfer to pots in the greenhouse. The survival of plants during acclimatization did not depend on the type or concentration of the auxin pretreatment. Acclimatized plants appeared healthy, without exhibiting any morphological abnormality or variation (Fig. 1d).

### Morphoanatomical analysis

SEM and histochemical analysis revealed that leaf indumentum of in vitro grown *M. pulegium*, like in other members of the Lamiaceae, consisted of non-glandular and glandular trichomes distributed over both adaxial and abaxial surface (Fig. 2a). Single, uniseriate non-glandular trichomes were sharply pointed, with tendency to lean toward the leaf apex, and consisted of one to three cells. The main types of glandular trichomes observed in *M. pulegium* leaves were peltate (Fig. 2b–e) and capitate (Fig. 2f–l), which were also found in the wild-growing plants (data not shown). The presence of these two glandular trichome types is a characteristic feature of Lamiaceae species (Ascensão et al. 1999).

In SEM micrographs, the surface of immature peltate glands appeared wrinkled, indicative of the close attachment of the cuticle to the secretory upper cell walls (Fig. 2c). During maturation, the gland surface becomes smoother as the secretory products accumulate within developing subcuticular space formed by detachment of the cuticle (Fig. 2d). Light micrographs showed that peltate trichomes consisted of a broad basal cell embedded in the epidermis, a short single-celled stalk with cutinized lateral walls, and a large round multicellular head (Fig. 2e). The glandular head of fully developed peltate trichome usually comprised eight secretory cells, arranged in a single disk (Fig. 2b). Such eight-celled glandular heads of peltate

trichomes are similar to those reported for *Salvia aurea* (Serrato-Valenti et al. 1997), *Plectranthus ornatus* (Ascensão et al. 1999) or *Mentha × piperita* (Turner et al. 2000).

Unlike morphologically rather similar peltate trichomes, capitate trichomes differ in their morphological characteristics in the way that reflects different secretory processes within trichomes and probably distinct functions (Ascensão et al. 1999). Two types of capitate trichomes, differing in size and structure, could be distinguished in *M. pulegium*. Type I capitate trichomes were positioned at an angle to the leaf surface and more abundant on the abaxial side (Fig. 2f). They were composed of one basal epidermal cell, short cutinized stalk and unicellular ellipsoidal head (Fig. 2g, h). In *M. pulegium* type I capitate trichomes, cuticle elevation was not observed and the secretory product accumulated within the secretory cell (Fig. 2g, h). Although type I capitate trichomes are known to be present in only a few Lamiaceae species, a comparable trichome type was also observed in *Micromeria croatica* (Kremer et al. 2012) and *Micromeria longipedunculata* (Kremer et al. 2014). Type II capitate trichomes displayed similar density on both sides. They were comprised of one conical, usually elongated basal cell, unicellular stalk and one apical secretory cell (Fig. 2i, j). Type II capitate trichomes in *M. pulegium* correspond to those described by Werker et al. (1985) for the Lamiaceae, and are very similar to those found in *Salvia officinalis* (Corsi and Bottega 1999) or *L. pedunculata* (Zuzarte et al. 2010). In mature trichomes, apical cell had well-developed round subcuticular space (Fig. 2k). Following rupture, the cuticle remained firmly attached to the cell wall in the basal part of the secretory cell, with cuticle detachment occurring only in its upper region (Fig. 2l).

### Histochemical analysis

The results of our histochemical analysis showed that the secretion of all types of *M. pulegium* glandular trichomes contained lipophilic substances. Although peltate trichomes have been generally assumed to contain the bulk of the essential oils produced by Lamiaceae, capitate trichomes of *M. pulegium* seemed to produce significant amounts of the essential oils.

In peltate trichomes, light-blue staining with Sudan Black B revealed the presence of lipids within the subcuticular space (Fig. 3a). The glandular secretion stained for neutral and acidic lipids with Nile Blue A gave positive reaction in the subcuticular space (pink staining) and head cells (intense blue staining, Fig. 3b). NADI reaction resulted in an intense dark-violet staining of the secretory product within subcuticular space, thus confirming the presence of terpenoid compounds in this trichome type

(Fig. 3c). Terpenoid secretion was shown to be restricted to peltate glandular trichomes in *Isodon rubescens* (Lamiaceae), which accumulate their secretory products in a large subcuticular space (Liu et al. 2012). The accumulation of the secretory product between cuticular sheath and the head cells has been described in many aromatic Lamiaceae (Werker et al. 1985; Serrato-Valenti et al. 1997; Zuzarte et al. 2010).

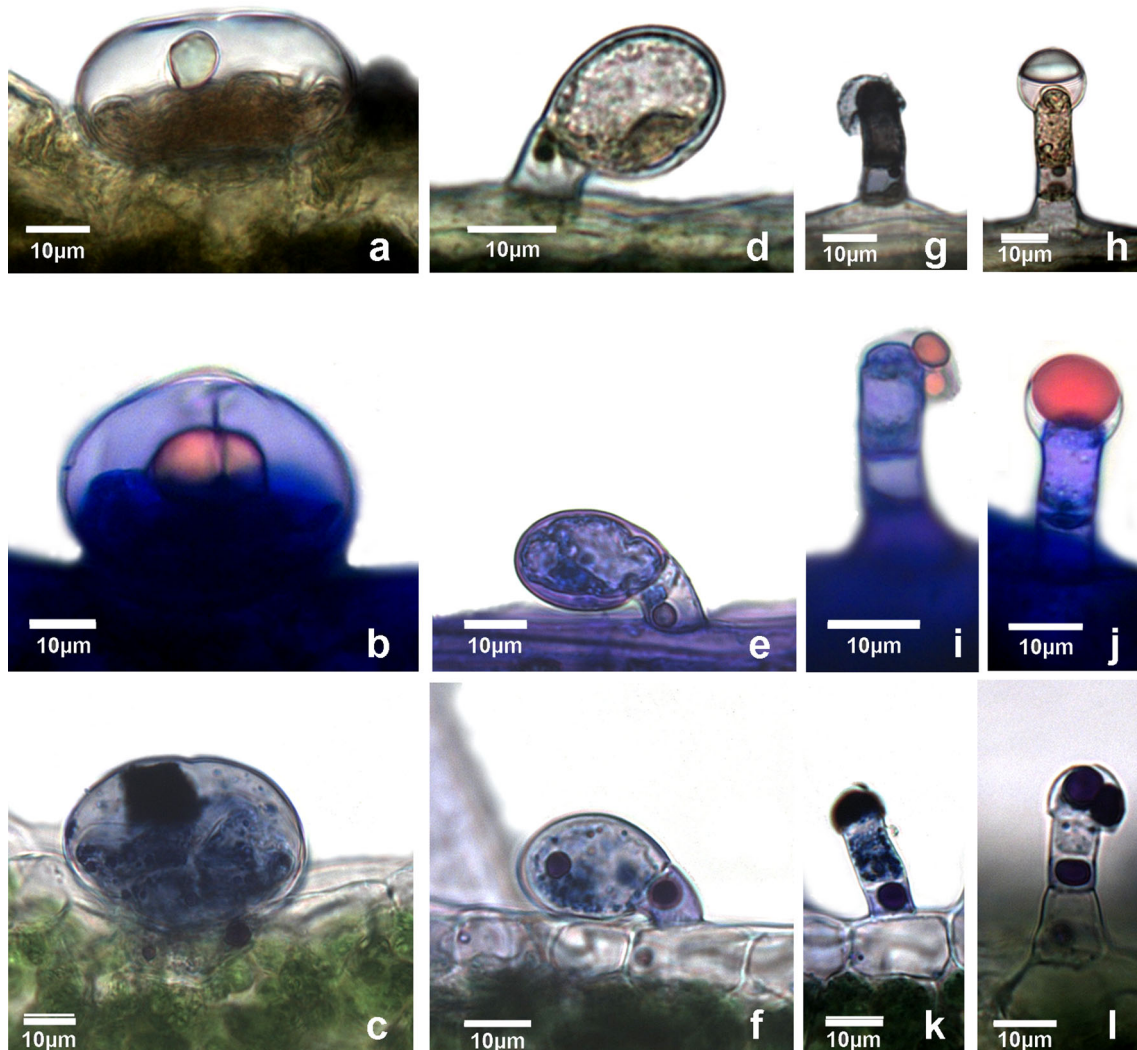
In type I capitate trichomes, head cell contained small amount of lipids (Fig. 3d), both neutral and acidic as suggested by positive reaction with Nile Blue A (Fig. 3e). Staining with NADI reagent confirmed the presence of essential oils in head and stalk cell (Fig. 3f). Lipophilic secretion of capitate trichome type II observed within the head cell (Fig. 3g) and the subcuticular space (Fig. 3h) stained blue with Sudan Black B. Secretory product within subcuticular space stained red with Nile Blue A, indicating the presence of neutral lipids (Fig. 3i, j). The NADI reaction resulted in an intense violet staining of type II capitate trichome, indicative of the presence of terpenoids (essential oils) in head and stalk cell (Fig. 3k), that became even more abundant in mature trichomes, as evidenced by dark-violet staining of the secretion accumulated in their subcuticular space (Fig. 3l). Zuzarte et al. (2010) showed that type II capitate trichomes of *L. pedunculata* were rich in lipidic and terpenic compounds, in contrast to type I capitate trichomes that were not involved in the production of the essential oils. Significant amounts of the essential oils were also found in the secretions of long-stalked capitate trichomes of *P. ornatus*, which also contained non-cellulosic polysaccharides (Ascensão et al. 1999).

Stalk cells of both type I (Fig. 3d) and type II (Fig. 3g, h) capitate trichomes of *M. pulegium* had thick cutinized lateral walls. In most glandular trichomes, the stalk cells contain lipophilic substances (Ascensão et al. 1999; Corsi and Bottega 1999). The thickenings of the cuticle on the lateral walls of the stalk cell, besides providing structural support, probably prevent the backflow of the secreted products into mesophyll tissue and regulate directional transport of metabolites to the glandular cells above (Ascensão et al. 1999; Gersbach 2002).

### Content and essential oil composition

In Lamiaceae, plant extracts secreted from glandular trichomes are a valuable source of biologically active compounds (Bassolino et al. 2015). Phytochemical analysis of wild-growing and micropropagated plants of *M. pulegium* revealed differences in both content and the composition of the essential oils obtained from different samples (Table 2). Micropropagated plants had lower yield of the essential oils than their parent plants, which yielded 0.47 % (v/w). The essential oil content in micropropagated plants





**Fig. 3** Histochemistry of in vitro leaf glandular trichomes of *Micromeria pulegium*. **a–c** Peltate trichomes. **a** Secretion stained light blue with Sudan Black B. **b** Positive Nile Blue A reaction in the subcuticular space (pink droplet + weak violet staining) and head cells (intense blue staining). **c** Secretion in the subcuticular space stained positively with NADI reagent. **d–f** Capitate type I trichomes. **d** Secretion in the head cell showing faint staining with Sudan Black B. Note intense staining of the stalk cell lateral walls. **e** Secretion stained with Nile Blue A. **f** Positive NADI reaction (dark violet) in the

head and stalk cell. **g–l** Capitate type II trichomes. Secretion stained dark blue in the head cell (**g**) and light blue in the subcuticular space (**h**) with Sudan Black B. Note intense staining of the head cell in **g**. Essential oils stained with Nile Blue A (pink droplets) accumulated in the subcuticular space of the early (**i**-) and late (**j**-) secretory-stage trichome. Terpenoids stained with NADI reagent (dark violet), accumulated in the subcuticular space of the head cell and in the stalk cell of the early (**k**-) and late (**l**-) secretory-stage trichome

ranged from 0.24 % (v/w) for plants grown on medium supplemented with 10 µM BA, to 0.38 % (v/w) for plants grown on PGR-free medium. The essential oil content was shown to depend on environmental factors, harvesting season and phenological stage (Hassiotis et al. 2014). Samples originating from natural and cultivated populations of *M. pulegium* were characterized by high amounts of the essential oils (0.8–1.4 %, v/w) at all stages of development (Slavkovska et al. 2013).

Differences in the essential oils composition were both quantitative and qualitative. In total, 12 compounds belonging to different terpenoid fractions were identified in the essential oils of the wild-growing plants, whereas in the essential oils of micropropagated plant material grown without PGRs or supplemented with BA 16 and 17 compounds were identified, respectively. The monoterpene fraction was dominant in all the analyzed samples (85.53–96.48 %), with the prevalence of oxygenated

**Table 2** The content and composition of the essential oils isolated from the aerial parts of *Micromeria pulegium* plants from natural populations (Wild-growing), and micropropagated plants (In vitro) grown on MS medium (MS) and on MS supplemented with 10  $\mu$ M BA (MS + 10BA)

	RI <sup>a</sup>	Wild-growing	In vitro	
			MS	MS + 10BA
Content (% v/w)		0.47	0.38	0.24
Compounds (%)	RI <sup>a</sup>			
Monoterpenoids				
Hydrocarbons				
$\alpha$ -Pinene	936	0.34	0.61	0.87
Sabinene	976	–	0.31	0.74
$\beta$ -Pinene	980	0.76	1.89	7.07
Myrcene	993	–	0.41	0.61
Limonene	1030	1.20	1.35	2.77
Oxygenated monoterpenes				
Ketones				
Menthone	1157	26.85	29.14	14.45
Isomenthone	1167	1.04	2.30	1.25
Isopulegone	1179	1.20	1.27	0.87
Pulegone	1243	60.07	44.57	50.77
Piperitone	1256	1.45	–	–
Piperitenone	1345	3.57	2.16	1.38
Oxides				
Piperitone epoxide	1258	–	4.91	2.29
Piperitenone oxide	1369	–	2.94	2.46
Alcohols				
Geranial	1273	–	0.71	–
Sesquiterpenoids				
Hydrocarbons				
$\beta$ -Caryophyllene	1423	–	0.93	1.28
Germacrene D	1485	–	3.62	6.06
Bicyclogermacrene	1500	–	1.07	2.13
cis-Calamenene	1525	–	–	0.59
Oxygenated sesquiterpenes				
Alcohols				
Spathulenol	1582	0.62	–	0.55
Oxides				
Caryophyllene oxide	1588	0.85	–	–
Non-terpenoid compounds				
Alcohols				
3-Octanol	997	0.26	–	–
Grouped components				
Monoterpenoids		96.48	92.57	85.53
Hydrocarbons		2.30	4.57	12.06
Oxygenated monoterpenes		94.18	88.00	73.47
Oxygenated monoterpenes of menthane type		94.18	87.29	73.47
Sesquiterpenoids		1.47	5.62	10.61
Hydrocarbons		–	5.62	10.06
Oxygenated sesquiterpenes		1.47	–	0.55
Other compounds		0.26	–	–
Total		98.21	98.19	96.14

<sup>a</sup> Retention index (RI) determined relative to a homologous series of *n*-alkanes C<sub>8</sub>–C<sub>40</sub> on HP-5 MS capillary column

monoterpenes of the menthane type (Table 2). The main constituent was pulegone (60.07 % in wild-growing plants, 44.57 % in micropropagated plants grown on PGR-free medium, 50.77 % in micropropagated plants grown on BA-supplemented medium), followed by menthone (26.85 % in wild-growing plants, 29.14 % in micropropagated plants grown on PGR-free medium, 14.45 % in micropropagated plants grown on BA-supplemented medium). Some of the volatiles belonging to monoterpenoids were found only in plant material obtained by micropropagation but not in wild-growing plants. These include sabinene and myrcene among monoterpene hydrocarbons, and geranial, piperitone epoxide and piperitenone oxide belonging to oxygenated monoterpenes. Predominance of monoterpene hydrocarbons and oxygenated monoterpenes in the essential oils of in vitro cultivated plants, in comparison with in vivo grown plants, was reported for *S. dolomitica* (Bassolino et al. 2015). The authors argued that the variation in the composition of the essential oils was the consequence of different ontological stage, where in vitro cultured plants are by definition considered as a juvenile-stage plant, with monoterpene accumulation being restricted to young leaf tissues (Croteau et al. 1981).

In our sample, the percentage of total sesquiterpenoids in micropropagated plants ranged from 5.62 % (PGR-free medium) to 10.60 % (BA-supplemented medium), and was considerably higher than in wild-growing plants (1.47 %). Sesquiterpene hydrocarbons in micropropagated plants accounted for the majority of detected sesquiterpenoids and were dominated by germacrene D, but were absent from the essential oils of wild-growing plants (Table 2). This contradicts the findings of Bassolino et al. (2015) for micropropagated *S. dolomitica*, where the percentage of total sesquiterpenoids strongly decreased in plantlets grown in vitro.

Monoterpene-to-sesquiterpene ratio was shown to be a stable character of *M. pulegium* essential oils and largely independent of environmental conditions (Slavkovska et al. 2013). Our study showed that sesquiterpene fraction was higher (lower monoterpene-to-sesquiterpene ratio) in micropropagated plants, especially those treated with BA, due to the presence of sesquiterpene hydrocarbons that were not found in parent plants from natural populations (Table 2).

In contrast to *L. pedunculata* where no qualitative changes in the volatile profile of micropropagated plants were observed (Zuzarte et al. 2010), a number of authors have reported on qualitative differences between wild-growing plants and those grown in vitro. In addition, secondary metabolite production can be drastically altered in response to different growth regulators in the culture medium (Affonso et al. 2009; Grzegorzczak-Karolak et al. 2015). Our results demonstrate that *M. pulegium*, both that gathered

from natural populations and cultivated in vitro, produces essential oil of relatively stable composition, yet at the same time suggest that the careful selection of the culture conditions could increase the accumulation of biomass and production of secondary metabolites, which could be employed to obtain essential oils for commercial use.

**Author contribution statement** DS designed and supervised the whole study and performed statistical analysis of the data. DS and ST performed tissue culture experiments. VS performed chemical analysis. BZ provided starting plant material and performed its botanical identification. DJ and SB performed microscopic analysis. BU wrote the manuscript and helped with data analysis and interpretation. All authors read and approved the manuscript.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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