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Essential Oil Contents and Micromorphological Traits of *Stachys iva* Griseb. and *S. horvaticii* Micevski (Lamiaceae)

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Abstract: Chemical composition of the essential oil (EO) (analysed by GC and GC/MS), the types and distribution of trichomes, and pollen morphology were investigated in *Stachys iva* Griseb. and *S. horvaticii* Micevski (Lamiaceae) growing in Republic of Macedonia. The essential oil of *Stachys iva* was characterized by a high concentration of oxygenated sesquiterpene (43.8%) of which caryophyllene oxide (34.2%) and spathulenol (8.3%) being the principal compounds, while hexadecanoic acid being a major component in the both *S. horvaticii* oil with percentages of 25.7% in the oil from locality Babuna River Gorge and 28.4 % in the oil from locality Rajec River Gorge. Non-glandular trichomes, and two type of capitate trichomes (type 1 composed of one basal epidermal cell, one stem cell and a unicellular head cell with subcuticular space; type 2 composed of one elevated basal epidermal cell, one stem cells, and a head composed of four, sometimes two small cells) were observed on leaves, calyx and the stem.

Keywords: Caryophyllene oxide; hexadecanoic acid; pollen; *Stachys iva*; *Stachys horvaticii*; trichomes.

1. Introduction

The genus *Stachys* L. (Lamiaceae) includes 300 (–450) annual or perennial herbs and small shrubs with a nearly worldwide distribution. They are occurring in temperate, subtropical, and tropical regions, except Malesia, Australia and New Zealand [1–3]. Fifty eight *Stachys* species have been described for Europe [1], and about twenty species for Republic of Macedonia (prof. Matevski, personal reference). *Stachys iva* Griseb. is a perennial herb with few ascending stems up to 40 cm high. It is an endemic species distributed in Macedonia and North Greece, on calcareous, stony slopes, rocks and cliffs [1]. *S. horvaticii* Micevski is a closely related endemic species distributed in

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Macedonia in the Vardar River Basin on calcareous, stony slopes, rocks and cliffs. It is distinguished from *S. iva* by the type of inflorescence, calyx teeth and the colour of flowers [4].

The family Lamiaceae is comprised of many species accumulating commercially important essential oil. Previous studies of the essential oils of *Stachys* species revealed their significant content of sesquiterpene compounds [5-9]. The oil of *Stachys palustris* L., *S. cretica* L., *S. germanica* L., *S. hydrophila* Boiss., *S. nivea* Labill., and *S. spinosa* L. besides sesquiterpenoidic compounds were characterized by carbonylic compounds, fatty acids and phenols [10, 11]. Despite these classes, in the ethyl acetate soluble fraction of *Stachys parviflora* from Pakistan was isolated diterpenoid – rosane type [12].

The oil of twenty-two different *Stachys* species from Antalia (Turkey) and *Stachys acerosa* Boiss. showed significant antimicrobial activity [5, 13]. Additionally, extracts of different *Stachys* species exert various pharmacological effects, like antioxidant, antitoxic and cytotoxic [7, 10, 12-15]. Thanks to these biological effects *Stachys* species have been used for centuries in traditional medicine as astringent, wound-healing, anti-diarrhoeal, anti-nephritic and anti-inflammatory agents [10, 16]. Also important for the possible medical use, were investigated the *in vitro* activity of five extracts of the aerial parts of *S. lavandulifolia* from Iran for the treatment of neurodegenetive diseases in relation to their metabolites profile [17].

Glandular trichomes are the site of essential oil biosynthesis, secretion and accumulation and their structure has been studied by several authors [18-23]. The present taxonomy ascribes great value to the structure and distribution of both secretory (glandular) and non-secretory trichomes [22, 24, 25]. In general, palynology gives valuable data about the origin, evolution and taxonomy of the species [26]. The pollen morphology of Lamiaceae was investigated by several authors [27-33].

The objective of this study is to gain insight into the essential oil contents and micromorphological features of endemic *Stachys iva* and *S. horvaticii*. To our best knowledge there is no data on essential oil contents or micromorphological traits of *S. horvaticii*. Therefore, this study represents a contribution to the chemotaxonomy of these endemic species.

2. Materials and Methods

2.1. Herbal material

Randomly selected samples of wild growing plants of *Stachys iva* Griseb. and *S. horvaticii* Micevski (Lamiaceae) from Republic of Macedonia were collected during the blooming period in July 2012. Samples of *S. iva* were collected in Pletvar near Prilep (GPS coordinates: 41°22'09" N; 21°39'06" E; 1020 m a.s.l. voucher no. HFK-HR-71-2012), while samples of *S. horvaticii* were collected in Babuna River Gorge near Veles (GPS coordinates: 41°41'02" N; 21°48'12" E; 175 m a.s.l.; voucher no. HFK-HR-74-2012) and in Rajec River Gorge near Kavadarci (GPS coordinates: 41°26'12" N; 21°52'06" E; 199 m a.s.l.; voucher no. HFK-HR-78-2012). Voucher specimens of herbal material were deposited in the Herbarium "Fran Kušan" (HFK-HR), Department of Pharmaceutical Botany with "Fran Kušan" Pharmaceutical Botanical Garden, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

The above ground parts of several dozen randomly selected plants were harvested from mature plants on a dry day and mixed to obtain randomly selected sample. Samples were air-dried for ten days in a well-ventilated room at 60% relative air humidity and room temperature (22 °C), single-layered and protected from direct sunlight. Dried aerial parts (100 g) were subjected to hydrodistillation for 3 h in Clevenger type apparatus. The obtained essential oil was dried over anhydrous sodium sulphate.

For micromorphological investigation of trichomes samples of leaves, flowers, and stems of five plants per locality were fixed in FAA (formalin/96% ethanol/acetic acid/water – 5/70/5/20; V/V/V/V) and later transferred to 70% ethanol.

2.2. Gas chromatography and mass spectrometry (GC, GC/MS)

The GC and GC/MS analyses were carried out with a Varian 3900 gas chromatograph (Varian Inc., Lake Forest, CA, USA) equipped with a flame ionization detector (FID), a 2100T mass selective detector (*Varian Inc*.), and a VF-5ms capillary column (30 m x 0.25 mm i.d., film thickness 0.25 mm; *Varian Inc*.). The oven temperature was programmed isothermal at 60 ºC for 3 min, rising from 60 to 246 ºC at 38º/min, and then held isothermal at 246 ºC for 25 min; injector temp., 250 ºC; FID temp., 300 ºC; ion-source temp., 200 ºC; carrier gas, He (1.0 mL/min); injection volume, 1 μL; split ratio, 1:20; ionization voltage, 70 eV; scanned mass range, 40 –350 amu.

2.3. Compound identification

The identification of the oil constituents was based on i) the comparison of their retention indices (RIs), determined relative to the retention times (R) of n-alkanes (C_8–C_{40}) on the VF-5ms column, with those listed in a homemade library, those reported in the literature, and/or those of authentic samples, ii) the comparison of their mass spectra with those reported in the literature [31] and those listed in the commercial mass spectral libraries Wiley 7 (Wiley, New York, NY, USA) and NIST02 (Gaithersburg, MD, USA), and iii) the co-injection with an authentic sample, wherever possible (see Table 1). The homemade library was created from authentic compounds obtained commercially and from the main components of many essential oils previously studied. The contents were calculated as mean values from the GC and GC/MS peak areas using the normalization method (without correction factors).

2.4. Micromorphological traits

For scanning electron microscopy (SEM) investigation samples (leaves, flowers, and stems) were transferred from 70% ethanol to 70% acetone, then further dehydrated (70%, 90%, and 100% acetone) and subjected to critical point drying using CO_2 as the drying medium (CPD030; Baltec). Dried samples were sputter coated with gold (Sputter Coater, AGAR) and investigated using a scanning electron microscope XL30 ESEM (FEI) with 20 kV acceleration voltages in high vacuum mode. The occurrence and frequency of the different trichomes types was qualitatively assessed (– missing, ± rare, + present, ++ abundant). Pollen from several flowers per plants (five plants per species) was removed from anthers after critical point drying and mixed to obtain random samples. The length of 30 pollen grains was measured. Light microscopical investigations were performed on hand-cut cross sections of fixed material using a Zeiss Axioplan 2 equipped with a camera (Axiophot 2). Common terminology was used in the description of micromorphology [32-34].

3. Results and Discussion

3.1. Gas chromatography and mass spectrometry (GC, GC/MS)

Aerial parts of wild-growing *Stachys iva* from Pletvar population were analyzed and forty-three components representing 82.3% of the total oil were identified. Total yield of the oil was 0.2%. The identified components listed in order of their elution from the VF-5ms column are given in Table 1 together with their percentages of the total mass fraction of the oil. Identified components are classified on the basis of their chemical structures in six classes. In essential oil of *S. iva* oxygenated sesquiterpenes was the dominant class (43.8%), with caryophyllene oxide being the principal compound (34.2%) followed by spathulenol (8.3%). In the literature data, the percentage of caryophyllene oxide in several other species of the genus *Stachys* varied from trace in *S. cretica* to 12.8% in the oil of *S. germanica* [5]. A sesquiterpene hydrocarbons was presented in a percentage of

24.7%, with *E*-caryophyllene (9.2%) and *δ*-cadinene (6.2%) as the main compounds. Monoterpene hydrocarbons, oxygenated monoterpenes, carbonylic compounds and hydrocarbons classes are found in concentrations of 13.8% (Table 1). Similarly to our results, spathulenol (8.1%), *E* -caryophyllene (9.3%), *δ*-cadinene (10%) were identified as one of major compounds in the oil of *S. iva* from Bislim-Kumanovo, Republic of Macedonia [38]. (Z)-nuciferyl isobutyrate is not representative in the here in investigation essential oil, but it appears to be the main compound in *S. iva* from Bislim-Kumanovo reporetd by Ristić et al. (2008) [38] and in diethyl ether extract reported by Lazarević et al. (2010) [39]. Variations in chemical composition of essential oil could be ascribed to environmental factors and genetic divergence.

Aerial parts of wild-growing *Stachys horvaticii* were analyzed on two different localities, Babuna River Gorge (BR) and Rajec River Gorge (RR). Total yield of the oil was 0.2% and 0.3% for oils from BR and RR, respectively. The identified components are given in Table 1. Both samples contained similar percentages of hydrocarbons (29% in BR, and 31.9% in RR) and sesquiterpene hydrocarbons (27.2% in BR, and 25.5% in RR). The high concentration of hexadecanoic acid (25.7% in BR, and 28.4% in RR) was also one of characteristics of *Stachys persica* Gmel. and *S. inflata* Benth. oil, where it accounts for 27.2% and 9.1%, respectively [40, 41]. Caryophyllene oxide (12.8% in BR, and 8.8% in RR) and *E*-caryophyllene (6.9% in BR and 6.3% in RR) were the next most abundant constituents in both *S. horvaticii* essential oils. The oil from RR contained higher concentration of monoterpene hydrocarbons (7.7%) than the oil from BR (1.4%), with *β*-pinene (5.1%) and *α*-pinene (2.6%) as the dominant components among this class (Table 1). In diterpene class was identified only manool with a very low rate of the both oil with percentages of 1.1 % in BR and 0.4 % in RR. According to Piozzi and Bruno (2009) [42] forty-five diterpenoids were detected, showing different skeleta (e.g. labdane, abietane, kaurane, pimarane) from thirty species and subspecies of genus *Stachys*. Manool was also indentified in essential oil of *Stachys candida* Bory et Chaub., *S. chrysantha* Boiss., *S. sylvatica* L., and *S. plumosa* Griseb. [9, 43, 44]. Group of carbonylic compounds represented 1.5% (oil from BR) and 0.7% (oil from RR) of the total oil.

The results showed a varying composition of the essential oils of the two investigated *Stachys* species. Therefore, it could be concluded that, based on essential oil composition, *S. iva* could be grouped into the oxygenated sesquiterpenes group, while *S. horvaticii* was the hydrocarbons chemotype. Producing of essential oil is one of the morphological and anatomical adaptations of much Lamiaceae plant which was formed in response to the specific conditions of plant life, as well as a hypersensitivity reaction to the temperature cross flow and water stress [45, 46]. Samples of *S. horvaticii* were collected in gorges of Babuna and Rajec rivers at altitudes of 175 and 199 m, respectively. Additionally, both gorges are under strong influence of sub Mediterranean climate. On the other hand, sample of *S. iva* was collected in mountain area at altitudes of 1020 m where the influence of sub Mediterranean climate is considerable lower. In general, secondary metabolites have important value for taxonomy but their occurrence more likely reflect adaptations to ecological conditions and particular life strategies embedded in a given phylogenetic framework and, therefore, as chemotaxonomic markers of *Stachys* species they have to be analysed carefully and critically.

3.2 Micromorphological traits

3.2.1 Trichomes

Type, occurrence and frequency of trichomes on leaves, calyxes, and stems of *Stachys iva* and *S. horvaticii* are shown in Table 2. In general, non-glandular and glandular trichomes could be observed on the investigated parts of both species. Different types of non-glandular (NG) trichomes are present in *Stachys* species [23, 24, 32, 47, 48]. NG trichomes in *Stachys iva* and *S. horvaticii* are long, multicellular, smooth, unbranched, uniseriate, folded at different levels, slightly raised above the epidermis by one elevated basal cell, and with more or less pointed tip. These trichomes were

extremely numerous and covered nearly all surfaces forming a very dense indumentum (Figure 1, a, b, i). According to Payne's plant hair terminology [35], NG trichomes could be noted as attenuate hairs. Bilušić Vundać et al., (2011) [23] noticed this trichome type also on *Stachys salviifolia* (Ten.) Rech. f. Comparable NG trichomes are also present in *Stachys ehrenbergii* Boiss. and *S. distans* Benth. [48]. On several *Stachys* species these NG trichomes reveal a warty surface due to cuticular micropapillae [23, 24, 48].

Table 1. Phytochemical composition (%), identification (%) and major groups of chemical components (%) of essential oil of *Stachys iva* and *S. horvaticii*.

Component	RI	Sample (yield in %)			Identification
		S. iva (0.2)	*S. horvaticii* (Babuna R.) (0.2)	*S. horvaticii* (Rajec R.) (0.3)	
Monoterpene hydrocarbons		**2.7**	**1.4**	**7.7**	
α-Pinene	938	0.1	0.2	2.6	R, MS, S
β-Pinene	982	0.3	1.1	5.1	R, MS
α-Terpinene	1016	–	0.1	-	R, MS
β-Phellandrene	1025	0.5	–	–	R, MS
Limonene	1032	0.7	–	–	R, MS
(Z)-β-Ocimene	1052	–	–	tr	R, MS, S
γ-Terpinene	1057	1.1	–	–	R, MS
Oxygenated monoterpenes		**8.4**	**7.3**	**9.3**	
trans -Linalool oxide (furanoid)	1088	0.1	0.1	1.4	R, MS, S
Linalool	1099	0.3	2.1	0.9	R, MS, S
trans-Pinocarveol	1147	–	1.2	0.4	R, MS
Camphor	1151	4.9	1.1	3.2	R, MS
Borneol	1176	0.9	–	–	R, MS, S
Terpinen-4-ol	1184	–	0.2	–	R, MS
α-Terpineol	1186	0.1	0.2	0.4	R, MS
Myrtenol	1197	–	–	0.2	R, MS
Verbenone	1204	–	0.4	–	R, MS
trans-Carveol	1215	0.6	1.1	1.9	R, MS
endo-Fenchyl acetate	1218	–	0.2	0.3	R, MS
Linalyl acetate	1252	0.2	0.3	–	R, MS

Bornyl acetate	1285	0.2	0.3	0.3	R, MS
α-Terpenyl acetate	1349	1.1	0.1	0.2	R, MS
Neryl acetate	1358	–	–	0.1	R, MS
Component	RI	*S. iva*	*S. horvaticii* (Babuna R.)	*S. horvaticii* (Rajec R.)	Identification
Sesquiterpene hydrocarbons		**24.7**	**27.2**	**25.5**	
δ-Elemene	1336	0.1	1.9	1.8	R, MS
α-Cubebene	1345	0.1	0.1	0.9	R, MS
α-Copaene	1377	0.1	0.3	0.3	R, MS
β-Bourbonene	1383	0.3	0.2	0.9	R, MS
β-Cubebene	1387	0.3	2.1	3.2	R, MS
α-Gurjunene	1407	0.9	–	0.1	R, MS
E-Caryophyllene	1424	9.2	6.9	6.3	R, MS, S
β -Copaene	1429	1.1	0.7	0.6	R, MS
trans-*α*-Bergamotene	1433	0.2	0.4	0.4	R, MS
(Z)-*β*-Farnesene	1454	0.3	0.2	0.4	R, MS
α-Humulene	1456	–	0.3	0.1	R, MS
allo-Aromadendrene	1465	1.9	1.7	1.8	R, MS
β-Chamigrene	1477	0.3	–	0.2	R, MS
Germacrene D	1481	2.1	3.8	1.5	R, MS
Viridiflorene	1496	0.1	0.5	1.3	R, MS
β-Curcumene	1514	–	1.2	1.6	R, MS
β-Bisabolene	1494	0.2	0.3	0.4	R, MS
γ-Cadinene	1513	0.4	2.2	–	R, MS
Bicyclogermacrene	1500	0.9	0.1	1.2	R, MS
δ-Cadinene	1521	6.2	4.3	2.5	R, MS
Oxygenated sesquiterpenes		**43.8**	**19.9**	**13.6**	
Spathulenol	1577	8.3	2.9	3.9	R, MS
Caryophyllene oxide	1581	34.2	12.8	8.8	R, MS, S

γ-Eudesmol	1632	0.3	1.6	0.2	R, MS
α-Cadinol	1655	0.4	0.6	–	R, MS
α-Bisabolol	1688	0.5	0.5	0.7	R, MS
α-Bisabolol oxide	1748	0.1	1.5	–	R, MS

Component	RI	*S. iva*	*S. horvaticii* (Babuna R.)	*S. horvaticii* (Rajec R.)	Identification
Carbonylic compounds		**0.1**	**1.5**	**0.7**	
Isobutyl hexanoate	1155	–	0.2	0.2	R, MS
Butyl n-hexanoate	1193	0.1	–	–	R, MS
Isoamyl hexanoate	1256	–	1.2	0.5	R, MS
β-Ionone	1487	–	0.1	tr	R, MS
Oxygenated diterpenes		–	**1.1**	**0.4**	
Manool	2056	–	1.1	0.4	R, MS
Hydrocarbons		**2.6**	**29.0**	**31.9**	
Eicosane	2000	–	0.3	0.1	R, MS, S
Hexadecanoic acid	1959	–	25.7	28.4	R, MS
Heneicosane	2100	0.8	0.2	–	R, MS, S
Docosane	2200	0.9	0.1	0.4	R, MS, S
Tricosane	2300	–	–	0.2	R, MS, S
Tetracosane	2400	–	0.4	0.1	R, MS, S
Pentacosane	2500	–	–	0.3	R, MS, S
Hexacosane	2600	0.8	2.3	2.2	R, MS, S
Heptacosane	2700	–	–	–	R, MS, S
Octacosane	2800	0.1	–	0.1	R, MS, S
Nonacosane	2900	–	–	0.1	R, MS, S
Total identified (%)		**82.3**	**87.4**	**89.1**	

RI, retention indices on capillary column VF5-ms; R, identification by comparison to literature [48], and/or home made library; MS, identification by NIST02 and Wiley 7 spectral databases; S, identification confirmed with reference compound; tr, traces (mean value below 0.1%); –, not identified.

Due to the very dense indumentums formed by the NG trichomes an evaluation of glandular trichomes was very difficult. However, combining scanning electron microscopy and light microscopy it was possible to identify two types of capitate trichomes on both species.

Type one capitate trichome (C1) is short, upright and composed of one basal epidermal cell (sometimes elevated), one stem cell and a unicellular, rounded head with a subcuticular space (Figure 1c, d and f). It is abundant on all investigated parts of the plants. On samples of *S. horvaticii* from the Rajec River Gorge occasionally comparable trichomes with two stem cells could be observed. Trichomes comparable to this type are present in *Stachys ehrenbergii* Boiss. [48] and *Micromeria fruticosa* (L.) Druce [18].Trichomes consisting of a basal cell, a distinct stem of one or two cells, and a unicellular, rounded head are also reported from *Salvia fruticosa* Mill. [18] and *Satureja* L. spp. [32].

Type two capitate trichome (C2) is only slightly bigger and composed of one elevated basal epidermal cell, a distinct stem cell and a rounded head that is composed of sometimes two but mostly four small cells (Figure 1e and g). Due to the small size (the head cell has a diameter of about 20 µm) and the smooth surface it was difficult to clearly distinguish both types of capitate trichome using SEM but they could be clearly distinguished using LM. C2 trichomes were less abundant than C1 trichomes. Trichomes comparable to this type consisting of a short unicellular or bicellular stalk with an apex composed of two secretory cells are known from *Stachys cretica* ssp. *vacillans* Rech. f., *S. distans* Benth., *S. neurocalycina* Boiss., *S. nivea*, and *S. palaestina* L. [48] and *S. recta* L. ssp. *serpentini* (Fiori) Arrigoni [25]. Short capitate trichomes composed of a basal epidermal cell, one stem cell and a secreting head of four cells are also known from *Stachys heraclea* All., *S. thirkei* C. Koch, *S. tymphaea* Hausskn., *S. alpina* L., *S. byzantina* K. Koch, *S. cretica* ssp. *salviifolia* (Ten.) Rech. f., *S. germanica* ssp. *dasyanthes* (Raf.) Arcang., *S. germanica* ssp. *germanica* [24], *S. recta* ssp. *recta* [25], *S. recta* ssp. *subcrenata* Vis., *S. alpina*, *S. salviifolia*, *S. sylvatica*, and *S. palustris* [23]. Giuliani and Maleci Bini (2008) [22] noticed small capitate trichomes composed of one epidermal cell, a neck or stalk cell and a head of 2–4 secreting cells in *Stachys alopecuros* (L.) Benth. ssp. *alopecuros*, *S. officinalis* L. ssp. *officinalis*, *S. germanica* ssp. *germanica*, *S. germanica* ssp. *salviifolia* (Ten.) Rech. f., *S. sylvatica*, *S. heraclea*, *S. plumosa*, *S. annua* (L.) L., *Prasium majus* L., *Sideritis romana* L., and *Scutellaria galericulata* L. Sessile capitate trichomes with one basal cell, very short neck cell and a secreting head of four cells were present in *Stachys officinalis* and *S. salviifolia* [23]. Finally, Bilušić Vundać et al. (2011) [23] noticed in *Stachys recta* ssp. *recta* short capitate trichomes inclined with the stalk cell nearly parallel to the surface. These trichome types, however, could not be observed on the here investigated species.

To summarize, non-glandular and two types of capitate trichomes occurring on *Stachys iva* and *S. horvaticii* are comparable to trichomes described for other *Stachys* species.

Table 2. Occurrence and frequency of trichomes on aerial parts of *Stachys iva* and *S. horvaticii*.

Specie	Type	Leaf		Calyx	Stem
		Adaxial	Abaxial	Outer	
S. iva	attenuate*	+++	+++	+++	+++
(Pletvar)	capitate C1	++	++	++	++
	capitate C2	+	+	+	+
S. horvaticii	attenuate	+++	+++	+++	+++
(Babuna	capitate C1	++	++	++	++
River)	capitate C2	+	+	+	+
S. horvaticii	attenuate	+++	+++	+++	+++
(Rajec	capitate C1	++	++	++	++
River)	capitate C2	+	+	+	+

Note. trichomes: +, present, ++, frequent; +++, abundant (forming a dense indumentum);
*attenuate, non-glandular hairs

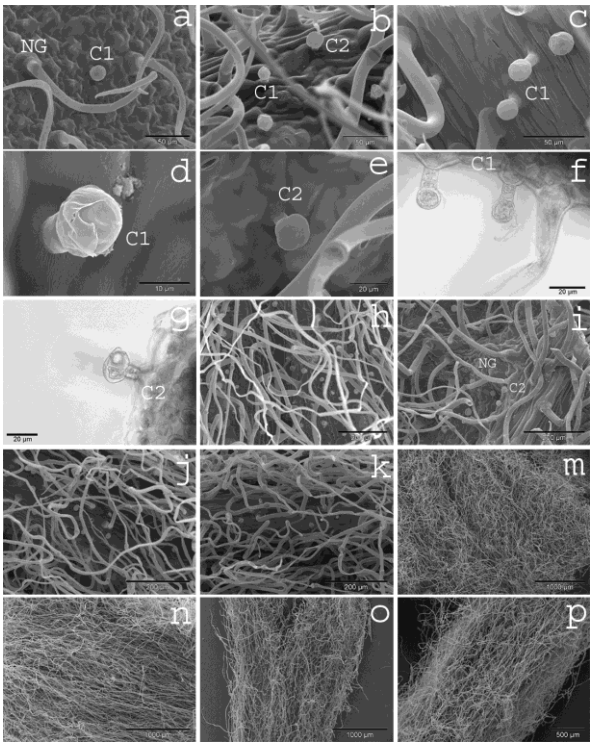

Figure 1. SEM micrographs of the different types of trichomes of *Stachys iva* (b, c, d, e, h, i, o) and *S. horvaticii* (a, f, g, j, k, m, n, p). Non-glandular trichomes (NG), type 1 capitate trichomes (C1) and type 2 capitate trichomes (C2), and their distribution on the adaxial (h, m) and abaxial (I, n) leaf surface, on the outer side of calyx (a, b, d, e, j, o), and on the stem (c, k, p). LM micrographs (f, g) of C1 trichomes with one and two stem cells on the leaf of *S. iva* (f), and C2 trichomes on the calyx of *S. horvaticii* (g)

3.2.2. Pollen

Pollen grains in *Stachys iva* and *S. horvaticii* are single (monad pollen) and isopolar with more or less spheroidal shape (Figure 2). According to Kremp's classification (1965) [49], pollen of *S. iva* belongs to small pollen (20–25 μm) with a polar axis of 24.58 ± 0.60 μm and equatorial diameter of 24.64 ± 0.47 μm. Minimum and maximum of polar axis are 24.0 μm and 25.5 μm, respectively, while minimum and maximum for equatorial diameter are 24.1 μm and 25.6 μm, respectively. According to the P/E ratio (0.99), it has spheroidal shape [36]. Pollen of *S. horvaticii* also belongs to small pollen with a polar axis of 23.76 ± 1.65 μm and equatorial diameter of 24.35 ± 1.54 μm. Minimum and maximum of polar axis are 23.3 μm and 27.9 μm, respectively, while minimum and maximum for equatorial diameter are 22.7 μm and 27.6 μm, respectively. According to the P/E ratio (0.98), it has spheroidal shape. Some other *Stachys* species, like *S. annua* L. [50], *S. cretica* [51], *S. germanica* [52], and *S. palustris* [53] have medium-sized pollen (26–50 μm).

Pollen of both species was similar without visible differences (Figure 2). The polar view in both species shows a circular shape with visible ends of the apertures, while equatorial outline is more or less circular. The pollen has three apertures (tricolpate), which are set in the equatorial pollen belt (zonocolpate). Apertures are long, rather wide, widest in the middle and gradually narrower towards the poles, with sharp edges, acute ends (Figure 2a and b), and ornamented membranes (Figure 2b and d). The apocolpium is quite small, while mesocolpium is rather large (Figure 2b and d). The exine has reticulate ornamentation (Figure 2c and f) with smooth muri surface. The lumina varied in size, but in general they are very small (microreticulate). In general, the appearance of pollen in *S. iva* and *S. horvaticii* are very similar to pollen of other *Stachys* species [50-53].

In this study chemical composition of the essential oil and micromorphological traits of trichomes and pollen of both *Stachys iva* and *S. horvaticii* are investigated. The chemical composition

of the essential oil revealed a high concentration of oxygenated sesquiterpenes in *S. iva*, while hydrocarbons were the major compounds in *S. horvaticii*. Investigations of plant trichomes showed the presence of attenuate non-glandular trichomes, and two types of capitate glandular trichomes on the leaves, the calyxes, and stems. The comparison between the morphological and phytochemical sets of data of two investigated *Stachys* species has not been reported before and therefore our results are a useful contribution to the taxonomy and better understanding of the interspecies relationships in the *Stachys* genus.

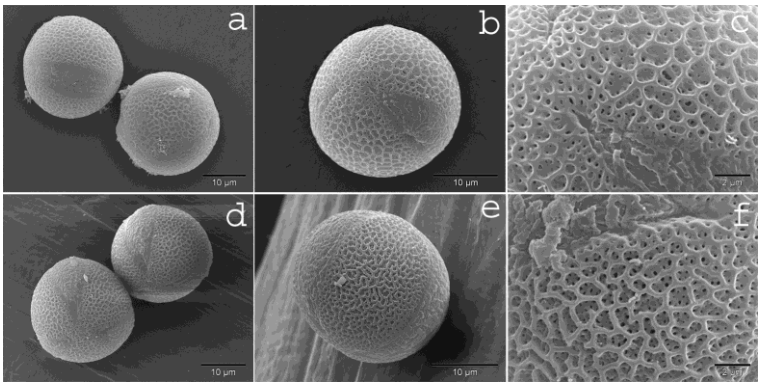

Figure 2. SEM micrographs of *Stachys iva* Griseb. (a, b, c) and *S. horvaticii* (d, e, f) pollen. Shape of pollen grains (a, d), apertures (b, e) and exine surface (c, f)

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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