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GLANDULAR HAIR ULTRASTRUCTURE AND ESSENTIAL OILS IN *SATUREJA SUBSPICATA* VIS. SSP. *SUBSPICATA* AND SSP. *LIBURNICA* ŠILIĆ

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Satureja subspicata spp. *subspicata* and *S. subspicata* spp. *liburnica*, collected during plant development, were studied comparatively with regard to the ontogenesis of their essential oils, chemical composition and glandular structure. The phytochemistry of essential oils isolated by hydrodistillation was analyzed, and 23 free volatile compounds were identified in all oils. The oils contained the monoterpenic phenols carvacrol and thymol in all phenological stages. The major component of both subspecies' oils was α-pinene, especially in the flowering period (52.9%, 42.6%), characterizing these plants as having the α-pinene chemotype. The glandular structure development of these xerophytic subspecies showed many ultrastructural changes preceding, during and after secretion. Metabolic changes were evident in the disc in cells in the pre-secretion stage, when the plant begins to produce terpenoids. The secretion gland head cells underwent a number of ultrastructural changes, among them the formation of a boundary wall. These changes resulted in an increase of surface tension and the accumulation of free volatile compounds in the subcuticular space of the gland head. In the post-secretion stage, all head cells began lysing and the basal cell was the only compact part of the gland, producing tannins as metabolic reaction to environmental stress.

Key words: Essential oil, glandular hair, *Satureja subspicata* Vis. ssp. *subspicata*, *S. subspicata* Vis. ssp. *liburnica* Šilić, ultrastructure.

INTRODUCTION

The rare species *Satureja subspicata* is represented by two subspecies, *Satureja subspicata* spp. *subspicata* and *Satureja subspicata* spp. *liburnica*, both growing in a limited area. Spp. *liburnica* is particularly rare and can be found in only one Croatian locality. The genus *Satureja* belongs to the Lamiaceae family, subfamily Nepetoideae, tribe Saturejeae, and comprises more than 200 species of herbs and shrubs (Pedersen, 2000; Cantino et al., 1992). The flora of Croatia has nine *Satureja* species, five subspecies and seven varieties. Three species grow wild in Dalmatia: winter savory (*Satureja montana* L.), wild savory (*S. cuneifolia* Ten.) and the investigated rare mountain savory (*S. subspicata* Vis.), which is a perennial semi-bushy plant growing in arid, sunny and rocky habitats up to 1200 m a.s.l. (Šilić, 1979).

Most Lamiaceae produce essential oils as secondary metabolites as part of their normal physiological function of growth, ecological function (interaction with the environment), development, or in response to pathogen attack or stress (Wink, 2003). The morphology of hair (glandular and non-glandular) has proved essential in taxonomic and ecological studies of this family (Dunkić et al., 2001; Werker, 1993; Gersbach, 2002). There are two main types of glandular hairs, peltate and capitate, carrying out different metabolic processes. Capitate hairs of different Lamiaceae species vary in stalk and head shape depending on the size of the subcuticular space (Ascensão et al., 1995). These structures produce a unicellular base, a unicellular stalk, and a 12-celled head in *Satureja thymbra* (eight-celled in *Mentha piperita*), and then commence filling the subcuticular oil storage cavity (Bosabalidis, 1990; Turner et al., 1999). The chemical composition of free volatile compounds depends upon intrinsic (genetic, growth stage) and extrinsic conditions such as climatic, seasonal, environment and distillation processes. They can be present as monoterpenes,

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sesquiterpenes, phenylpropanes and their derivatives. The pathways of monoterpene, sesquiterpene, and diterpene biosynthesis can be divided into several stages of glandular hair secretion. The morphology and ultrastructure of secreting glandular hairs (e.g., leucoplast, ER, mitochondria, Golgi) have been examined in only a few Lamiaceae species such as *Mentha piperita, Mentha spicata, Salvia glutinosa, Salvia pratensis, Salvia officinalis, Monarda fistulosa, Origanum dictamnus, Teucrium scorodonia, Teucrium scorodonia* and *Nepeta racemosa* (Ascensão et al., 1997; Turner et al., 2000a). The terpenoid synthesis enzymes are operationally soluble enzymes localized to the cytosol (sesquiterpene synthesis) or plastids (monoterpene synthesis and diterpene synthesis) (Bohlmann et al., 1998). Cheniclet and Carde (1985) and Skubatz et al. (1995) suggested that there is a relationship between the structure of secretory cell plastids, SER or RER, and their monoterpene synthesis. When essential oil contains many monoterpenes, the gland contains leucoplasts devoid of ribosomes and thylakoids. The rough endoplasmic reticulum plays the main role in biosynthesis, accumulation and secretion of sesquiterpenes in the cell of *Sauromatum guttatum*. Several species within the genus *Satureja* have potential therapeutic activity, especially in traditional medicine, mostly due to their essential oils, which are mixtures of many volatile constituents (Lawrence, 1992). The oil components of *S. subspicata* possess antimicrobial properties and are therefore a potential source of antimicrobial ingredients for food and the pharmaceutical industry (Skočibušić et al., 2006).

This paper is the first contribution on ultrastructural complexes of different organelles involved in metabolic production of terpenes and phenylpropane derivatives during glandular ontogenesis. The second part of our investigation compares the free volatile compounds of *S. subspicata* ssp. *subspicata* and *S. subspicata* ssp. *liburnica*. The developmental dynamics of glandular structures, together with oil secretory processes, have a direct bearing upon secondary metabolite production.

MATERIALS AND METHODS

LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

The first leaf shoots of *Satureja subspicata* Vis. ssp. *subspicata* were collected in April 2005 on Kozjak Mt. (near Split, Croatia), and *S. subspicata* ssp. *liburnica* Šilić on the south slope of Velebit Mt. Voucher specimens are deposited in the herbarium of the Faculty of Natural Science and Kinesiology of the University of Split [No. FNSMKST 2005: 13 (A, B, C), 14 (A, B, C)]. Hand-cut sections from leaves preserved in 50% ethyl alcohol were observed and photographed with an Opton Axioskop MC 63A light microscope.

Tissue sections ~2 mm^2 were cut from leaves and fixed in 2% w/w glutaraldehyde for 30 min. After fixation the tissue was washed twice with 0.1 M phosphate buffer, 30 min per wash. Sections were post-fixed in 1% w/w OsO4 for 2 h, followed by a further three 15 min washes in distilled water. Sections were dehydrated in a water/acetone series (30, 50, 60, 70, 80, 90 and 100% w/w acetone) for 10 min each and infiltrated with Durcopan ACM (Fluka) and 100% w/w acetone (1:1) for 2 h. The sections were embedded in flat modules with fresh resin and polymerized at 64°C for three days. Semi-thin sections (2 μm) were cut with an RMC Boeckeler Instrument MTX, stained with Toluidine Blue, examined by light microscopy and photographed. Ultrathin sections (60–75 nm) were cut and collected on Formvar-coated copper slot grids. The mounted ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). After drying, the sections were examined and photographed with an FEI Morgagni 268D transmission electron microscope (TEM).

ISOLATION OF ESSENTIAL OILS

Essential oils from both subspecies of *S. subspicata* were isolated from fresh plant material (100 g) collected prior to flowering (leaves and stalks; July), in the course of flowering (flowering tops, leaves and stalks; September) and after flowering (leaves and stalks; November). They were subjected to hydrodistillation for 3 h in a Clevenger apparatus. The essential oils obtained were dried over anhydrous sodium sulphate, and 2 μl of each was used for analysis.

Gas Chromatography (GC) and Mass Spectrophotometry (MS) Analysis of Oils

GC analyses were performed with a Varian 3900 gas chromatograph equipped with a flame ionization detector (FID) and a CP Wax 52 CB low-bleed polyethylene glycol capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). Other conditions: carrier gas, hydrogen; flow rate, 0.8 ml/min; injection type, split, 1:10; temperature program, 50–270°C at 5°C/min. The quantitative analysis of oil components employed peak area normalization measurements.

GC-MS analyses of the volatile compounds used a Hewlett-Packard GC-MS system (GC 5890 Series II; MSD 5971A) coupled directly to a fused-silica HP–20 M polyethylene glycol column (50 m × 0.2 mm i.d., 0.2 μm film thickness). Other conditions: carrier gas, helium (1 ml/min); temperature program, 4 min isothermal at 70°C, then 70–180°C at 4°C/min, then held isothermal 10 min; injection port temperature, 250°C; sample components ionized in

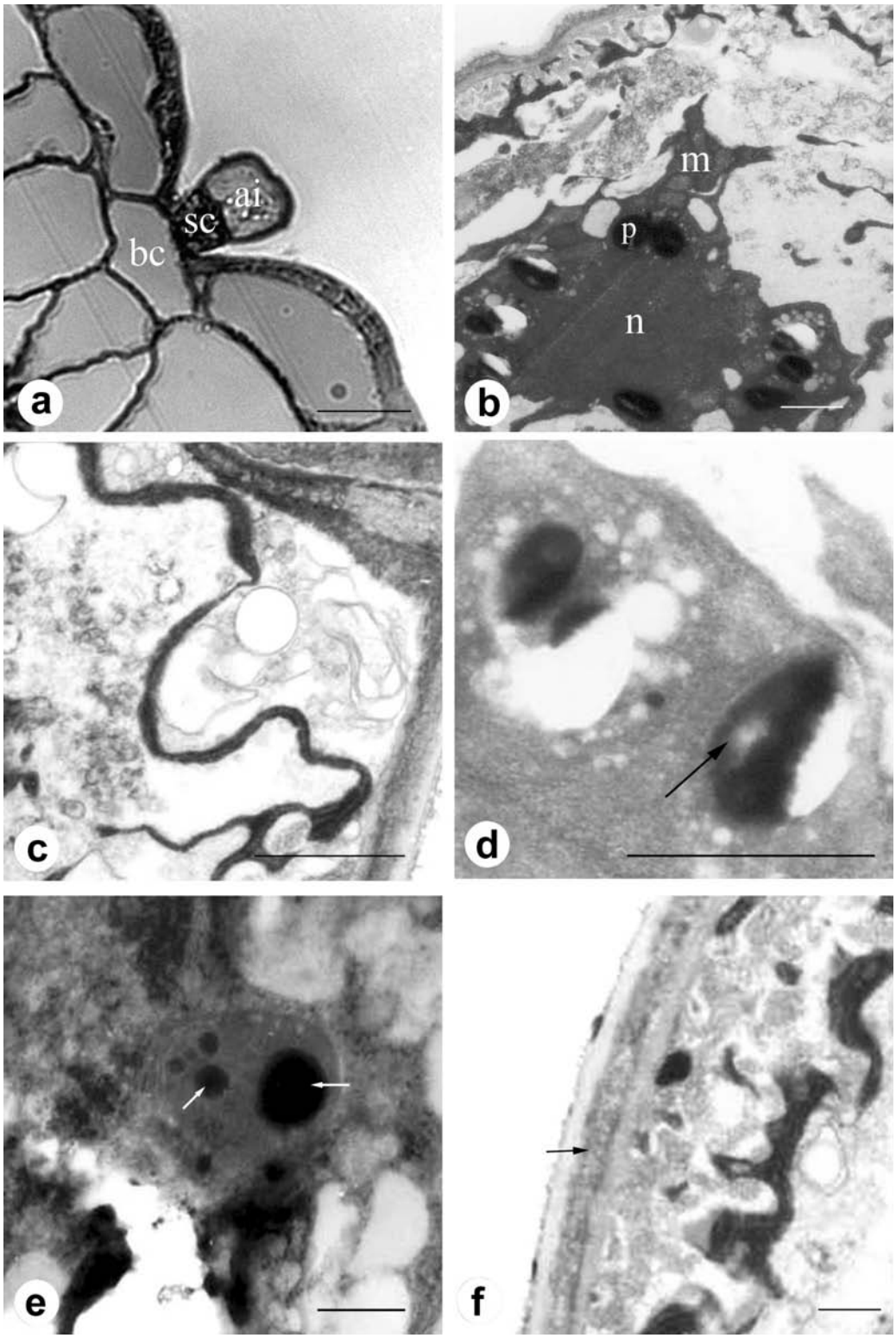

Fig. 1. Pre-secretion stage of *Satureja subspicata* gland. (**a**) Cross section of gland with bc – basal cell, sc – stalk cell, ai – apical disc, by LM. Bar = 10 μm, (**b**) Central part of apical disc. n – nucleus; p – proplastid; m – mitochondria, by TEM. Bar = 2 μm, (**c**) Vesicle-like structure with ER of apical cell, by TEM. Bar = 1 μm, (**d**) Vacuoles with osmiophilic essential oil (arrow), by TEM. Bar = 1 μm, (**e**) Proplastid with plastoglobuli (arrows), by TEM. Bar = 1 μm, (**f**) Three-layer cell walls with a middle pectin layer (arrow), by TEM. Bar = 2 μm.

EI mode (70 eV). The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing a homologous series of C_8-C_{22} *n*-alkanes (Adams, 2007). The individual constituents were identified by comparison of their retention indices with those of known compounds retrieved from the literature (van Den Dool and Kratz, 1963), and also by comparing their mass spectra either with those of the known compounds or with the Wiley mass spectral database.

RESULTS AND DISCUSSION

As in most Lamiaceae plants, the surface of *Satureja subspicata* leaves has glandular hairs present. The distribution and density of peltate hairs are related to enlargement of the leaves; they are very abundant over the abaxial surface of the young leaf and decrease with the increase in leaf area. Gland initiation is prolonged, and a large number of glands are distributed randomly over the abaxial and adaxial leaf surfaces; glandular hair production ceases during leaf enlargement, so the final number of hairs is established at an early stage of leaf development (Ascensão et al., 1995). Both mature and meristematic regions appear during leaf development. As long as cell divisions occur in the leaf, new glandular hairs may be produced. Peltate glandular hair development during ontogenesis in *S. subspicata* is divided into three phases: pre-secretion, secretion and post-secretion.

The pre-secretion stage is the time of cell division and growth, and early oil production. Future glands arise as epidermal protuberances dividing asymmetrically to produce a basal cell and an apical cell. Further divisions of the apical cell produce one stalk cell and one head cell (Fig. 1a). In almost all young secretory glands, completely cutinized walls are assumed to block the back-flow of secreted material to the neighboring tissue through the apoplast (Hallahan et al., 2000). The central part of head cell ultrastructure contains a large nucleus, with numerous plastids, mitochondria, both types of ER, vesicle-like structures and a few small vacuoles around it (Fig. 1b). The vesicle-like structures (Fig. 1c) are surrounded by lipophilic secretion; osmiophilic (dark part) lipid material is also evident in vacuoles (Fig. 1d). The first step of monoterpene biosynthesis has been specifically localized to the colorless plastids (leucoplasts) because the universal acyclic precursor geranyl diphosphate arises from primary metabolism at this site (Turner et al., 2000, 2000b). The proplastids from the stalk cell are large, often appearing roughly spherical, and have large plastoglobuli (Fig. 1e). The wall of the apical cell has three layers with a central pectin lamella, which is fully developed prior to the phase pre-

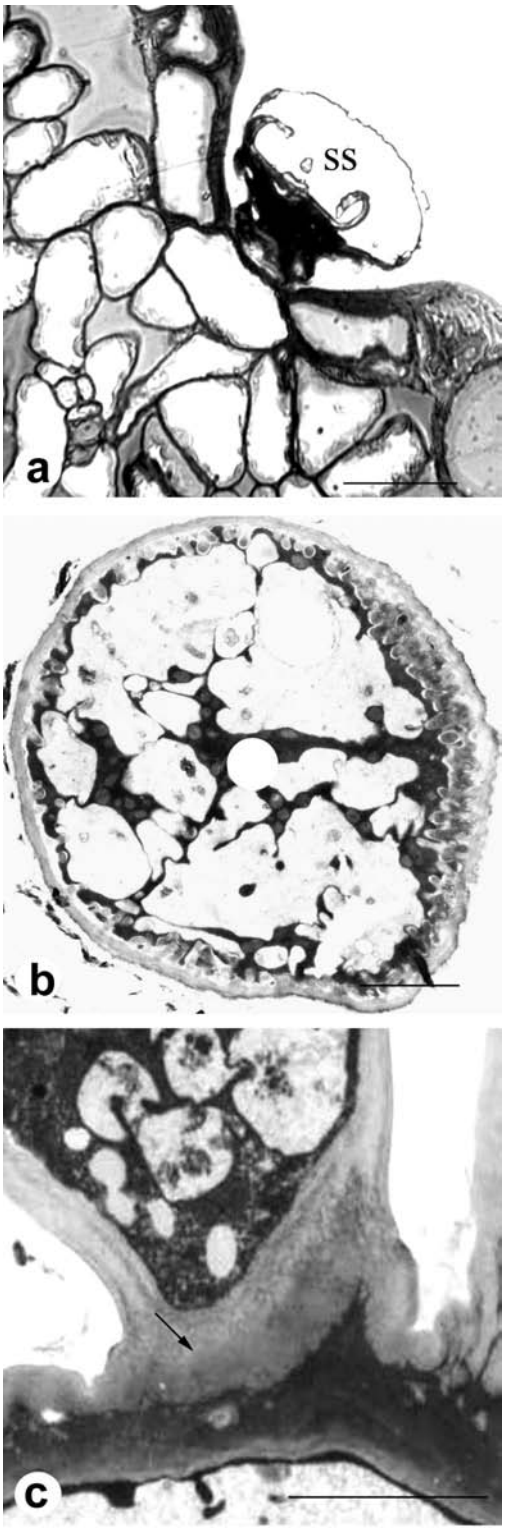

Fig. 2. Secretion stage of *Satureja subspicata* gland. (**a**) - Subcuticular space (SS) and aged peltate hair, by LM. Bar = 10 μm, (**b**) Large vacuoles containing dispersed fibrillar material in contact with cell boundary wall of head cell, by TEM. Bar = 2 μm, (**c**) Cutinized cell wall (arrow) separating the stalk and basal cells, by TEM. Bar = 2 μm.

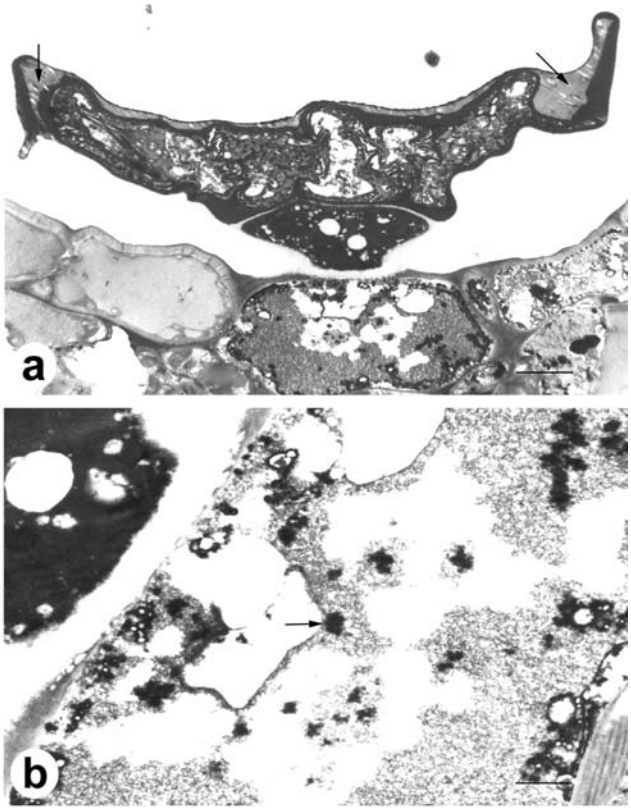

Fig. 3. Post-secretion stage of *Satureja subspicata* gland, by TEM. (**a**) Subcuticular space losing stored oil (arrows). Bar = 1 μm, (**b**) Basal cell containing large vacuole with tannins (arrows). Bar = 2 μm.

ceding secretion (Fig. 1f). Invaginations of the inner cell wall system protruding into the cytoplasm shorten the oil transport distances between the cytosolic compartments and subcuticular storage space (Bourett et al., 1994).

The secretion stage is a time of anticlinal cell division in apical discs, producing a twenty-celled head disc and eight peripheral cells, four of which radially surround the central cells, as in *Satureja thymbra* and *S. cuneifolia* (Bezić et al., 2001). The next step in this phase is detachment of a thick cuticle from the outer 12-celled head walls to form an extensive extra-cellular subcuticular storage space filling with essential oil. The secretory glands form depressions of the epidermis, undulating the leaf surface (Fig. 2a). The secretory gland head cells contain large, ultrastructurally altered vacuoles, now losing their surface tension (Fig. 2b). The vacuoles are in close contact with plastids and the cell boundary wall. A highly cutinized cell wall (Fig. 2c) separates the stalk cell from the basal cell; that is the moment when a gland has finished its secretion phase.

Post-secretion, glands are vacuolate and contain dispersed lipid material. In this phase the subcuticular space loses the stored oil through a rupture of the cuticle made by the pressure of the secreted substance. After the cuticle ruptures, the head glandular cells become cup-shaped (Fig. 3a). The essential oil is now released into the leaf epidermis and mesophyll. When the glands are lysing, the broken leaf surface is susceptible to microbial invasion, water loss and isolation. This is characteristic of all xerophytic plants growing under extreme environmental conditions. At this stage of ontogenesis the basal cell is the only compact part of the gland after the secretion stage. This cell differs from the basal cell before and during the secretion stage; it is heavy and contains a large central vacuole and tannins (Fig. 3b). Tannins are plant stress metabolites. Their synthesis is induced by outside factors and their role is to increase plant resistance to heat and infections. Besides lignin, which is part of the apical cell wall, tannins are another plant phenolic polymer, localized only in the basal and epidermal cells of the post-secretion gland. Phenol-containing cell areas are electron-dense (Nielson and Griffith, 1978).

For phytochemical analyses, essential oils isolated by hydrodistillation of both subspecies of *S. subspicata* were subjected to detailed GC and GC-MS analyses to determine their chemical composition. The essential oils were isolated at the different vegetative stages from *S. subspicata* ssp. *subspicata* and *S. suspicata* ssp. *liburnica*. The yields were highest during the flowering period for all the investigated plants (Tab. 1). All of the identified 23 free volatile compounds were similar in the two subspecies, and the major component was the monoterpenic hydrocarbon α-pinene, 52.9% for ssp. *subspicata* and 42.6% for ssp. *liburnica*. A high concentration of α-pinene is not characteristic of other *Satureja* species, but is specific to woody plants such as the genus *Pinus* (Bruneton, 1995) and some *Salvia* species. For sage, the possibility of nonenzymatic conversion to α-pinene from other compounds was excluded, indicating direct cycling from geranyl pyrophosphate to α-pinene (Glambiel and Croteau, 1982). The investigated *Satureja* plants are perennial woody plants with chemical and xerophytic adaptations to environmental stress, classified as having the α-pinene chemotype.

The phenols carvacrol and thymol were among the main constituents of the oils, while their precursors γ-terpinene and *p*-cymene were not identified before flowering of *S. subspicata* ssp. *subspicata*. The highest share of *p*-cymene was during flowering, when the share of thymol and carvacrol was lowest. Thymol and carvacrol cannot be considered separately, since they both have a very close biosynthetic relationship with their precursors γ-terpinene and

TABLE 1. Composition (%) of essential oils from fresh leaves of *Satureja subspicata* Vis. ssp. *subspicata* and *S. subspicata* Vis. ssp. *liburnica* Šilić before (July), during (September) and after (November) flowering; numbers in parentheses are global averages

No.	Component	RI HP20M	*Satureja subspicata* ssp. *subspicata* Month (yield %)			*Satureja subspicata* ssp. *liburnica* Month (yield %)		
			July (1.7)	September (2.0)	November (0.2)	July (0.9)	September (1.8)	November (0.6)
1.	α- pinene	1032	12.8	52.9	13.5	15.3	42.6	16.7
2.	myrcene	1174	–	5.0	5.9	2.1	3.9	9.9
3.	limonene	1201	8.1	4.5	4.2	16.2	5.2	11.5
4.	(E)-β-ocimene	1246	–	1.1	–	0.5	–	t
5.	γ-terpinene	1255	–	5.1	9.4	0.6	2.2	8.1
6.	*p*-cymene	1261	–	16.7	4.7	1.2	8.5	6.6
7.	*allo*-ocimene	1351	2.7	1.0	1.2	–	2.2	1.5
8.	1-octen-3-ol	1394	1.0	1.8	2.2	2.4	0.7	0.6
9.	*t*-sabinene hydrate	1474	–	–	0.9	0.1	–	–
10.	β-bourbonene	1535	1.7	–	1.1	1.1	0.2	–
11.	linalool	1537	5.5	0.6	3.9	4.4	3.9	0.9
12.	terpinen-4-ol	1611	–	0.2	–	0.1	1.9	2.5
13.	β-caryophyllene	1612	0.5	–	t	–	–	1.6
14.	germacrene D	1705	0.2	0.4	4.1	–	2.1	4.3
15.	borneol	1719	–	0.9	4.2	–	–	2.2
16.	geraniol	1724	–	–	3.4	6.2	0.5	3.8
17.	geranyl acetate	1729	–	0.3	4.7	3.7	1.5	2.6
18.	myrtenol	1804	0.6	–	0.7	1.1	1.7	0.9
19.	nerol	1808	–	–	3.1	2.1	–	–
20.	eugenol	2141	5.2	1.6	0.2	2.5	4.7	–
21.	spathulenol	2144	2.3	–	1.0	–	–	2.8
22.	thymol	2198	13.2	2.9	2.1	19.5	5.8	4.6
23.	carvacrol	2239	15.3	0.7	16.8	13.5	7.6	9.8

RI – retention indices sorted according to HP-20 M; t – Peak area < 0.1%; – Not identified

p-cymene. The oil of *S. subspicata* ssp. *liburnica* was also characterized by a high content of limonene (16.2>5.2<11.5%), geraniol (6.2>0.5<3.8%) and their derivative geranyl acetate (3.7>1.5<2.6%). Other important monoterpenes in both investigated subspecies were myrcene, linalool and eugenol. The presence of eugenol and other *p*-hydroxyphenylpropanes in the Lamiaceae indicates an association with lignin biosynthesis (Stahl-Biskup et al., 1993). In the investigated plants this synthesis was directly connected with the lignin concentration in the cell wall and the production of tannins in glands after secretion.

In the Lamiaceae family, oil-poor species are generally known to be rich in sesquiterpene hydrocarbons such as germacrene-D and β-caryophyllene. Germacrene-D has also been detected as the main compound in *Satureja coerulea* from Turkey, and β-caryophyllene was one of the main components in the endemic species *Satureja isophylla* from Iran (Sefidkon et al., 2004). *S. subspicata* ssp. *subspicata* and *S. subspicata* ssp. *liburnica* were poor in oil (0.2% and 0.6%) in the period after flowering, just when it was rich in germacrene D, known for its pheromone activity. The sesquiterpene compounds were present in small quantities in all phenological stages of the investigated plants (Tab. 1).

Glandular hair density and oil secretion in *S. subspicata* leaves depend on the stage of development. The hairs are very abundant on young leaves, but their metabolic production decreases progressively as the leaves develop. Then they contain less

lipophilic material and the glands are particularly well suited for study of their ultrastructural organization.

Satureja subspicata grows in at higher elevations than any other *Satureja* species in Croatia. The investigated plants show xerophytic metabolic activities resulting from oxidative stress in glandular tissue, and increasing the plants' defenses. These ultrastructural changes and changes in the metabolic activity of terpenoid production during glandular development are described here for the first time.

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