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Structure and chemistry of glandular trichomes of selected *Micromeria* and *Clinopodium* species (Lamiaceae): *in vitro* culture approach

Original Article

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Abstract:

Many of the species belonging to the Lamiaceae family are considered aromatic plants due to the presence of glandular trichomes, which have a distinct ability to synthesize, secrete or store large amounts of specialized metabolites that play a crucial role in mediating the plant–environment interactions. These compounds often have marked bioactive properties, rendering a commercial value to the plants that produce them. A number of biological effects have been associated with the main monoterpenoids detected in investigated *Micromeria* spp. and *Clinopodium* spp. essential oils. One alternative for the production of these bioactive metabolites is *in vitro* plant tissue culture. The present study was initiated to investigate the effects of *in vitro* culture on the secretion of leaf glandular trichomes, the main structures involved in the essential oil production. The glandular indumentum was studied by means of light microscopy and scanning electron microscopy in an attempt to correlate the phytochemical traits with the glandular trichome morphotypes of selected Lamiaceae species.

Key words:
glandular trichomes, histochemistry, Lamiaceae, micropropagation, morphology

Apstrakt:

Struktura i hemija žlezdanih trihoma odabranih vrsta rodova *Micromeria* i *Clinopodium* (Lamiaceae): primena kulture *in vitro*

Mnoge vrste familije Lamiaceae su označene kao aromatične biljke zahvaljujući prisustvu žlezdanih trihoma, koje odlikuje karakteristična sposobnost da sintetišu, luče ili skladište velike količine specijalizovanih metabolita koji posreduju u interakciji između biljaka i njihove životne sredine. Ova jedinjenja često imaju značajna bioaktivna svojstva, koja biljkama koje ih produkuju daju komercijalnu vrednost. Veliki broj bioloških efekata dovodi se u vezu sa glavnim monoterpenoidima detektovanim u etarskim uljima ispitivanih vrsta *Micromeria* spp. and *Clinopodium* spp. Kultura biljaka in vitro predstavlja jednu od alternativa za proizvodnju ovih bioaktivnih metabolita. Prikazano istraživanje je započeto sa ciljem da se ispitaju efekti kulture in vitro na sekreciju žlezdanih trihoma listova, osnovnih struktura uključenih u proizvodnju etarskih ulja. Žlezdani pokrivač je proučavan primenom svetlosne i skenirajuće elektronske mikroskopije, u pokušaju da se ustanovi korelacija između fitohemijskih odlika i morfotipova žlezdanih trihoma odabranih biljnih vrsta familije Lamiaceae.

Ključne reči:
histohemija, Lamiaceae, mikropropagacija, morfologija, žlezdane trihome

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Secretion refers to the complex phenomena of separation of secreted substances from the protoplast and their removal either to the plant surface or into internal spaces, or their accumulation in some compartment of the cell (Evert, 2006). The secreted substances are produced by the secretory tissues of diverse structure and topographic position that occur in most vascular plants. Glandular trichomes are the most recently evolved secretory structures found on the surface of about 30% of all vascular plants. They vary in their structure, in the chemical composition of the substances they produce and the mode of their secretion, and in their function.

Many of the species belonging to the Lamiaceae

family are considered aromatic plants due to the presence of glandular trichomes, a valuable source of biologically active volatile compounds. Glandular trichomes in Lamiaceae can produce a variety of specialized metabolites, among which terpenoids are particularly well represented and have been used by humans in a variety of industries (Tissier et al., 2013). Glandular trichomes secreting lipophilic substances generally consist of a group of glandular cells at the apex of a stalk of one or more cells in length. The storage compartment of glandular trichomes is part of the glandular cell, or cells, which are metabolically active. The present taxonomy ascribes great value to the structure (shape, cell number) and distribution of trichomes, as well as to the type of the secretion and its storage, and mode of release (Giuliani & Maleci Bini, 2008). Lamiaceae members are characterized by a great variety of trichomes. From a functional viewpoint and in accordance with their mode of secretion, the glandular trichomes of Lamiaceae species can be classified into two main types, peltate and capitate trichomes (Hallahan, 2000; Werker, 2000).

Peltate trichomes have common characteristics in structure and morphology across genera and mostly produce and store biogenic volatile or semi-volatile organic compounds related to plant abiotic or biotic stress responses (Corsi & Bottega, 1999; Turner et al., 2000; Machado et al., 2006). They are the main sites of essential oil production and storage, as revealed by their ultrastructure and histochemistry (Werker, 1993; Turner et al., 2000). Peltate trichomes are considered long-term trichomes, given that the accumulation of the secreted material continues during the growth of the organs that bear them (Werker, 1993). There are various types of capitate trichomes that differ significantly in both structure and size (Werker, 1993; Werker et al., 2000), and whose primary function is thought to be the repelling of the pests (Lin & Wagner, 1994). Capitate trichomes are specialized to produce and store a large amount of diterpenes and a wide array of nonvolatile or poorly volatile compounds that are directly exuded onto trichome surface and are particularly active at the early stages of organ development. They are considered short term trichomes because the secretory materials are extruded to the outside soon after their production, and the entire process of secretion is soon terminated (Werker, 1993).

The plant family Lamiaceae, one of the largest among the dicotyledons, comprises more than 7200 species across approximately 240 genera. The vast number of genera and species, especially those placed in subtribe Menthinae (tribe Menthae, subfamily Nepetoideae), are spices and medicinal herbs of great economic importance (Brauchler et al., 2010). The genus *Micromeria* Benth. comprises more than 70 perennial herbs, sub-shrubs and shrubs, rarely annual herbs, distributed throughout the temperate belt (Harley et al., 2004; Erhardt et al., 2014). *Micromeria* spp. are more or less aromatic species producing a small quantity of essential oils dominated by various terpene compounds. Closely related genus *Clinopodium* L. comprises *ca*. 100 (Braüchler et al., 2010) to 135 (Erhardt et al., 2014) perennial herbs, including taxa that were recently transferred from the polyphyletic genus *Micromeria* sect. *Pseudomelissa* based on molecular and morphoanatomical evidence (Braüchler et al., 2006). *Clinopodium* species are distributed world-wide, with the Mediterranean being the main center of species diversity and sectional diversity and are also aromatic. They produce variable quantities (depending on the sample collection site) of essential oils dominated by oxygenated monoterpenes. Essential oils of *Micromeria* and *Clinopodium* species exhibit substantial antimicrobial (Marinković et al., 2002; Duru et al., 2004; Šavikin et al., 2010; Vuko et al., 2012) and antioxidant activities (Vladimir-Knežević et al., 2011), thus protecting the plant from pathogen attacks. It is for these properties that the aboveground plant parts of some *Micromeria* and *Clinopodium* species are used for medical, insecticidal, herbicidal, and culinary purposes (Duru et al., 2004; Šavikin et al., 2010; Vladimir-Knežević et al., 2015).

Plant sources are increasingly being exploited for new drug development, and there is a growing interest in validating traditional medicines and herbal remedies (Verpoorte, 2000). Natural sources of bioactive compounds used as pharmaceuticals, agrochemicals, flavors, fragrances, food additives and biopesticides are often endangered due to increasing industrial demands. Plant cell, tissue, and organ cultures have emerged as potential sources of structurally complex and high-value natural products, especially if the plant source material is an overexploited, slow-growing, or low-yielding plant. Through the application of various *in vitro* approaches and strategies, plant cell, tissue, and organ culture techniques permit manipulation of growth and production of medicinally or commercially important plant metabolites in the microenvironment of *in vitro* cultures, independently of geographical or seasonal variation (Isah et al., 2018). A valuable method for the multiplication of selected genotypes and chemotypes of many medicinal and aromatic plants is *in vitro* propagation from wild-growing plants through axillary shoot formation (**Fig. 1**).

The present study was initiated to investigate the effects of *in vitro* culture on the secretion of leaf

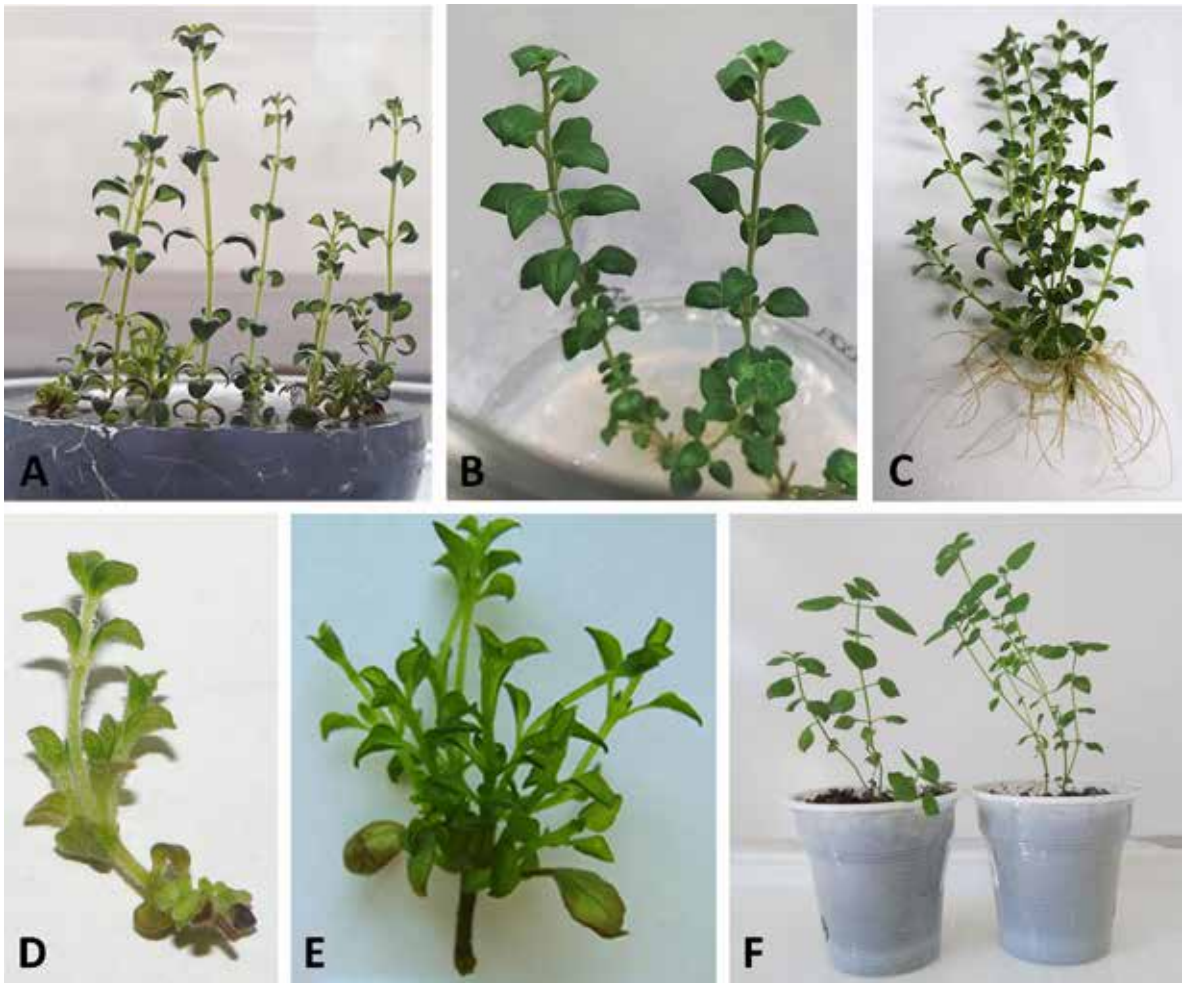

Fig. 1. Micropropagation of selected *Micromeria* and *Clinopodium* species. *In vitro* plantlets of *Micromeria graeca* cultured on plant growth regulator-free medium after 4 weeks of culture (**A-C**); note numerous axillary shoots and well-developed adventitious roots in **C**. *In vitro* plantlet of *Micromeria croatica* (**D**). Axillary bud proliferation of *Clinopodium pulegium* (**E**). Acclimatized *C. pulegium* plantlets regenerated via axillary shoots (**F**).

glandular trichomes, the main structures involved in the essential oil production, in an attempt to correlate the phytochemical traits with the glandular trichome morphotypes of selected Lamiaceae species. To that aim, we carried out observations on micromorphology, morphoanatomy, and secretion of the glandular trichomes in shoot cultures of *Micromeria croatica* (Pers.) Schott, *Micromeria graeca* (L.) Benth. ex Rchb., *Clinopodium pulegium* (Rochel) Bräuchler and *Clinopodium thymifolium* (Scop.) Kuntze.

General aspects of plant trichomes

The plant trichomes are specialized uni- or multicellular epidermal projections of diverse form, structure, and functions, which cover most surfaces of most plants (Esau, 1953). They display greatly variable morphology depending on the organ and the species, which has often been used in plant classification (Wagner, 1991). In Lamiaceae, the type, size and density of trichomes vary within genera, within species, and between different organs of the same plant, but most differences are species-specific and can often be of taxonomic significance (Bhatt et al., 2010). The physiological functions of trichomes are diverse, as their morphological and mechanical features (size, shape, density, orientation) influence many aspects of plant physiology and ecology (Wagner et al., 2004). Two general types of trichomes can be discerned: non-glandular trichomes, which mainly differ in their morphology, and glandular (secreting) trichomes, which typically differ in the substances that they secrete.

Non-glandular trichomes play an important role in mechanical defense against biotic and abiotic stresses, and generally do not produce or secrete phytochemicals. These trichomes are diverse in morphology, anatomy and microstructure. Non-glandular trichomes are abundant in the early phases of leaf development, but their density decreases with

leaf maturity (Corsi & Bottega, 1999; Ascensão et al., 1999).

Glandular trichomes are the specific sites for the biosynthesis, accumulation and excretion of secondary metabolites, i.e. pest- or pollinator-interactive chemicals that are stored or volatilized from the plant surface (Keene & Wagner, 1985; Turner et al., 2000; Werker et al., 2000). They not only accumulate and store what is often phytotoxic secretory material in a compartment that is virtually outside the plant body, but also position these compounds as an apparent first line of defense at the surface of the plant (Wagner, 1991). Glandular trichomes contain cells that are highly specialized for the biosynthesis and secretion of copious amounts of particular secretory products (Lange & Turner, 2013).

Glandular trichomes are the most recently evolved secretory structures, and are characteristic feature of many angiosperms (Fahn, 1988; Lange & Turner, 2013). They are suggested to have developed phylogenetically from non-glandular trichomes, because both trichome types are initiated and develop similarly up to the stage of a three-celled primordium, after which the differences between the two types begin to appear (Fahn & Shimony, 1977).

From a functional viewpoint, based on the mode and timing of secretion, glandular trichomes can be classified into two types: long-term glandular trichomes, which gradually accumulate their secretion in a subcuticular space, and short-term glandular trichomes, which start and end their secretion rapidly (Werker, 1993). The former corresponds to peltate trichome morphotype, whereas the latter correspond to capitate trichome morphotype. Peltate trichomes do not exhibit considerable variations in their basic morphology, although they can differ significantly in their size (Ascensão et al., 1999; Corsi &Bottega, 1999; Turner et al., 2000; Machado et al., 2006; Stojičić et al., 2022). Various types of capitate trichomes differ significantly in both structure and size (Werker et al., 1985; Ascensão & Pais, 1998; Kolb & Müller, 2004; Amrehn et al., 2014). Apart from morphology, peltate and capitate trichomes also differ in metabolites that they produce. Besides volatile or semi-volatile stress-related organic compounds that are secreted and stored in extracellular storage space, capitate trichomes produce a wide array of nonvolatile or poorly volatile compounds that are directly exuded onto trichome surface.

Trichome types, functions, and distribution in Micromeria and Clinopodium spp. in vitro

The aerial parts of *in vitro* grown plants of all examined species are covered with indumentum that appears colorless to the naked eye and consists of non-glandular and glandular trichomes. The most conspicuous are numerous sharply pointed, unbranched non-glandular trichomes, which are particularly elongated and abundant on leaf petioles and midribs (**Fig. 2A**). Uniseriate non-glandular trichomes with warty surfaces (**Fig. 2B**) are found on both leaf sides and are especially abundant along the margins and veins and at the leaf base (**Fig. 3A**). Their morphology and distribution on plants *in vitro*

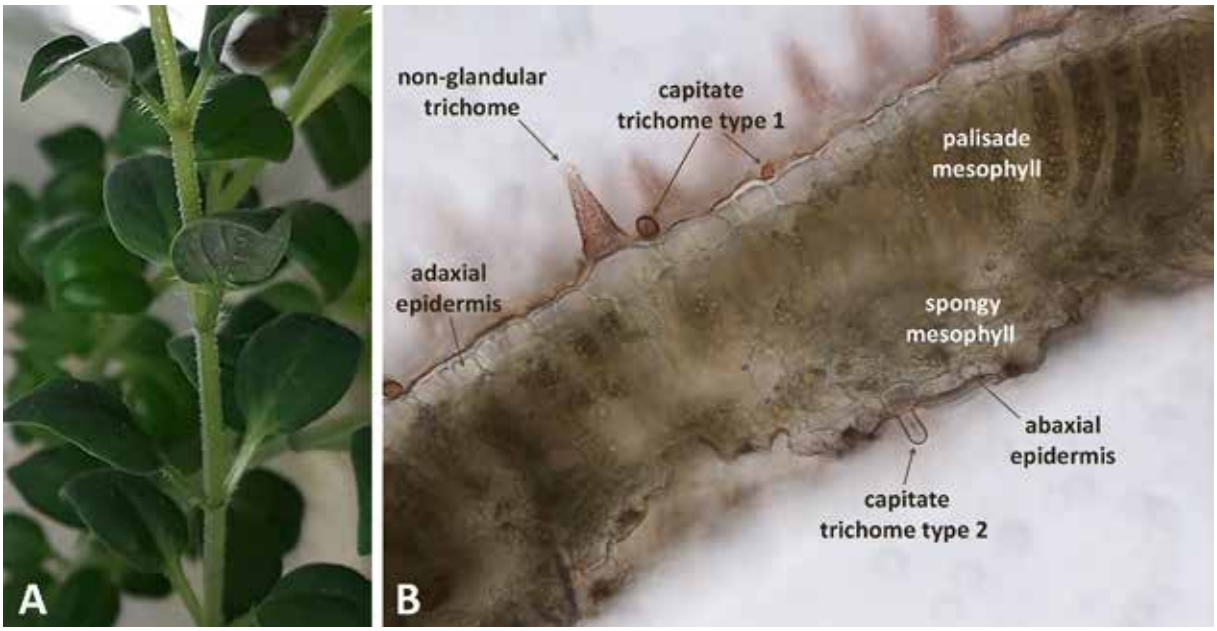

Fig. 2. Stem and leaf indumentum of *in vitro* grown *Micromeria graeca* plantlets (**A**). Cross section of pubescent leaf, showing many non-glandular and glandular trichomes on both leaf surfaces (**B**)

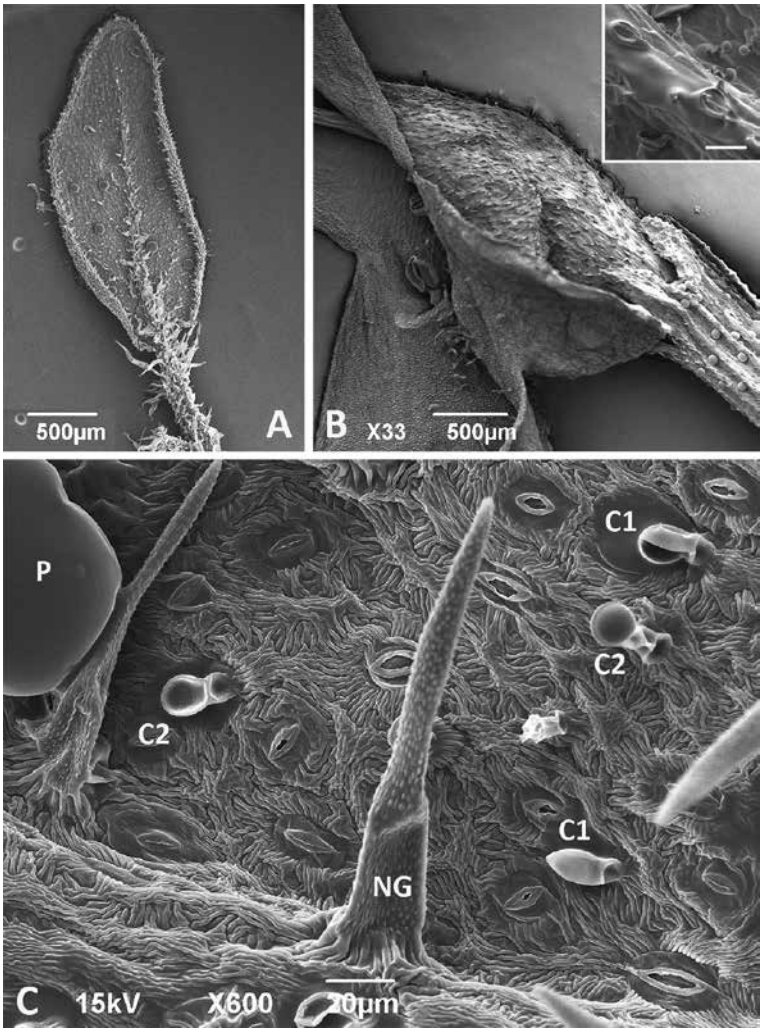

Fig. 3. SEM micrographs showing distribution and types of trichomes on vegetative and reproductive organs of *in vitro* grown plants. Young, not fully developed leaf of *Micromeria graeca*, with many non-glandular trichomes at the leaf margins and along the veins on the abaxial surface (**A**). Mature glandular trichomes on calyx of *Clinopodium thymifolium*. Inset: detail of B, showing C2 trichomes at full secretion (*Bar*=25 µm) (**B**). Abaxial side of fully developed leaf of *Micromeria croatica*. P – peltate trichome; C – capitate trichomes type 1 (C1) and type 2 (C2); NG – non-glandular trichome (**C**)

fully correspond to wild-growing plants (Kremer et al., 2012; Dunkić et al., 2017). The length of these trichomes varies from very short on the leaf lamina, to very long on leaf petioles and stems, which display the densest indumentum of non-glandular trichomes.

Glandular trichomes are constant features of many plant species and develop over the aerial vegetative and reproductive plant organs without external stimuli (Fahn, 1988). Although covering all the aboveground organs of *in vitro* propagated plants, glandular trichomes are particularly abundant on the leaves, where they appear on both sides but at different proportions and are particularly concentrated on the intervein areas in all examined species (**Fig. 3**).

Trichome production and maturation is limited to short periods early in leaf development (Hülskamp et al., 1994). In all examined *in vitro* plants, glandular trichomes are initiated very early during leaf ontogeny, with trichome initials and young developing trichomes present already on the youngest leaf primordia (**Fig. 4**). Glandular trichomes arise from a single expanding protodermal cell undergoing a periclinal division. Peltate and capitate trichomes morphotypes cannot be easily distinguished at their inception, both being first discernible as protruding protodermal cells with an asymmetrical cytoplasmic distribution (**Fig. 4A**). After enlarging and extending above from the leaf surface, the expanded protodermal cell is partitioned by a periclinal, asymmetrical cell division, resulting in the two-celled stage trichome comprised of an apical, more meristematic cell and a basal, more vacuolated cell (**Fig. 4B**). Slightly older, three-celled stage trichomes are further partitioned by periclinal cell divisions, separating an apical initial and a stalk cell, atop a vacuolated basal cell (**Fig. 4C**).

The apical initial may remain unicellular, as is the case in capitate trichomes of all investigated species or give rise to multicellular secretory head of peltate trichomes, as a result of several rounds of anticlinal divisions, depending on the species. Further changes on the ultrastructural level occur in accordance with the chemistry of glandular trichome secretion (Ascensão & Pais, 1998; Turner et al., 2000; Giuliani & Maleci Bini 2008; Amrehn et al., 2014; Uzelac et al., 2015).

The main types of glandular trichomes observed in micropropagated plants are peltate and capitate, which is a characteristic feature of Lamiaceae species (Ascensão et al., 1999). Peltate trichomes are less abundant than capitate trichomes in all examined species. They are present on both leaf surfaces but are more abundant on the abaxial side (**Fig. 3**, **Fig. 5**). Two types of capitate trichomes, differing in size and structure, could be distinguished in all examined species: type 1 (C1) and type 2 (C2) capitate trichomes (**Fig. 3C**). Both capitate types are found on both abaxial and adaxial surfaces of leaves, on stems and

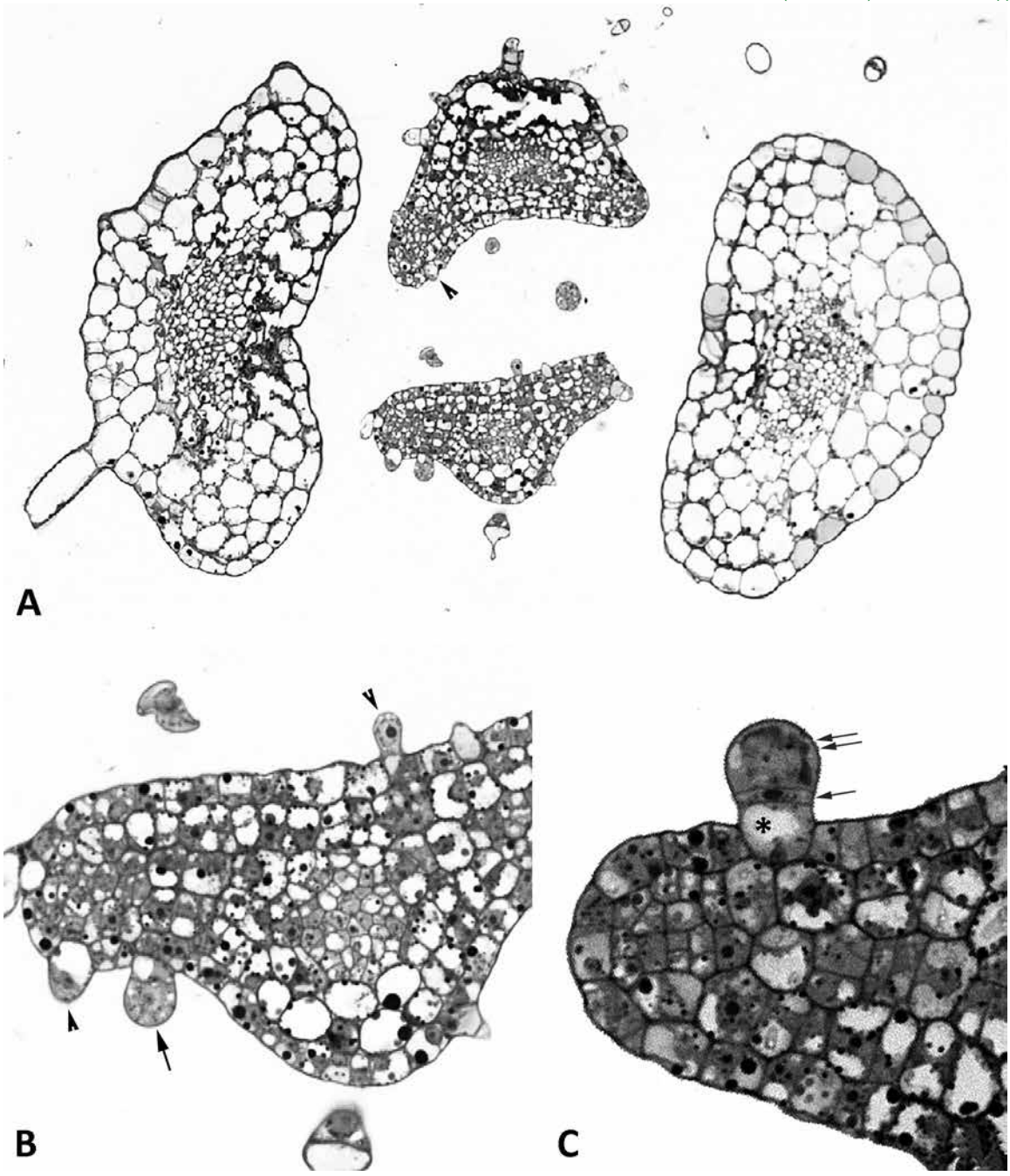

Fig. 4. Glandular trichome initiation on leaves of *in vitro* cultured *Micromeria croatica*. **A** Transverse section through shoot apex, showing two youngest pairs of leaf primordia with trichomes at different developmental stages, mostly on the abaxial side. **B** Detail of **A**. Note trichome initial (*arrowhead*) adjacent to two-celled stage trichome (*arrow*) on the youngest leaf primordium. **C** Early-stage glandular trichome following periclinal division of protodermal cell, comprising a more vacuolated basal cell (*asterisk*) and two densely cytoplasmatic cells; later during ontogeny, a smaller proximal meristematic cell (*arrow*) will give rise to the stalk cell, whereas larger apical cell (*double arrow*) will give rise to glandular head

on the abaxial side of calices (**Figs. 2**, **3B-C**, **5B**). On leaves of *in vitro* grown plants, C1 trichomes were the most abundant trichome type, randomly distributed over the entire surface (predominantly abaxial). C1 trichomes are the most commonly occurring capitate trichome type in Lamiaceae, reported in nearly all the species examined (Werker et al., 1985; Ascensão et al., 1999; Mota et al., 2013). They were found in all investigated *Micromeria* and *Clinopodium* sp. plants *in vitro*, as well as in their wild-growing counterparts (Kremer et al., 2012; Marin et al., 2013; Dunkić et al., 2017; Kremer et al., 2021). Compared

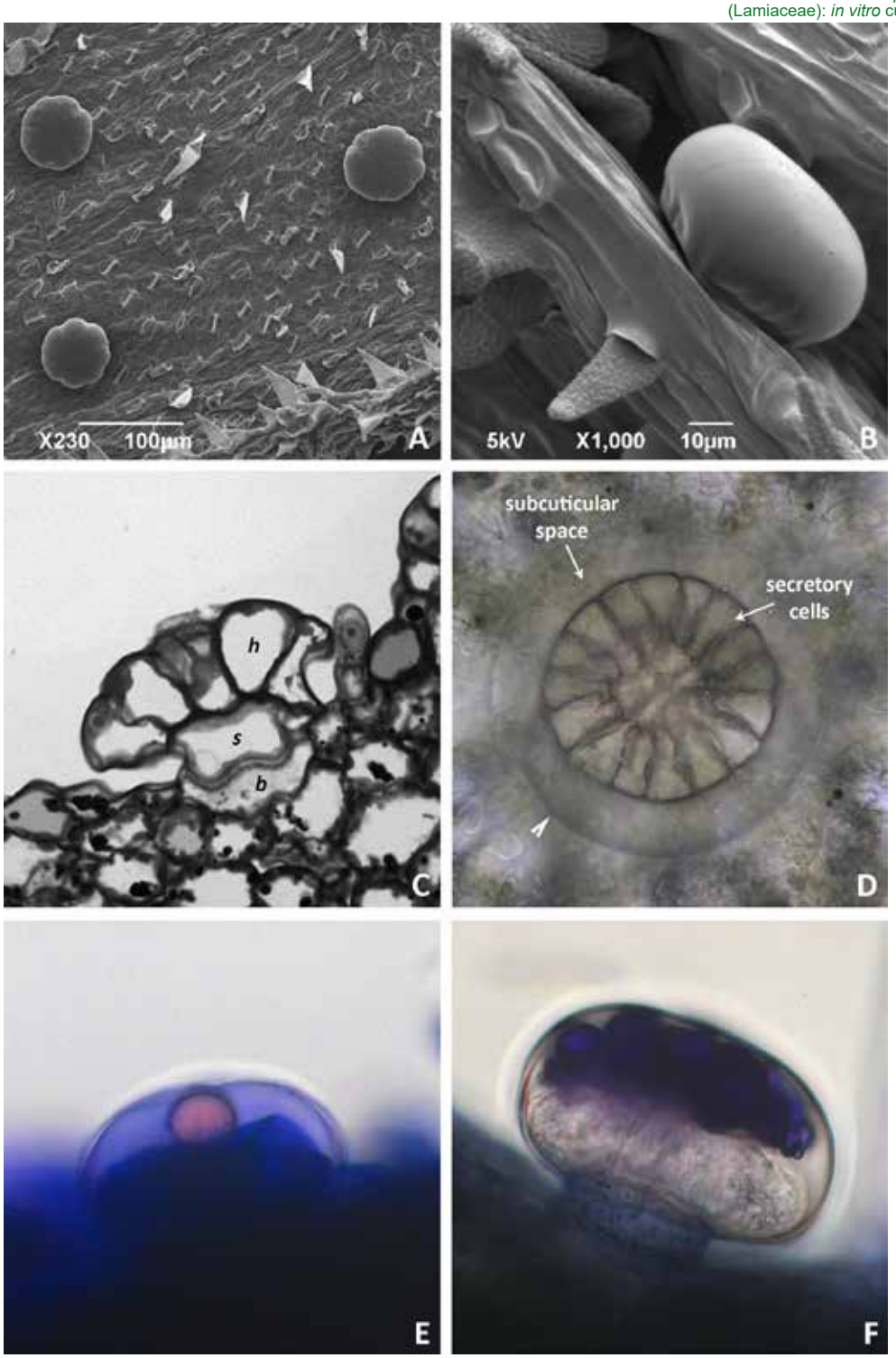

Fig. 5. Peltate trichomes of *in vitro* grown plants. **A-B** SEM micrographs showing peltate trichomes at different secretory stages: immature, presecretory to early secretory stage trichomes of *Micromeria graeca* (**A**) and fully mature, secretory stage trichome of *Clinopodium thymifolium* (**B**). Longitudinal section of post secretory stage peltate trichome of *Micromeria croatica* (**C**). Note multicellular secretory head (*h*), comprising 4 central cells surrounded by 12 peripheral cells arranged in a shield, subtended by unicellular stalk (*s*) and vacuolated basal cell (*b*). Upper view of glandular head of fully developed peltate trichome of *M. graeca*, with 4 central cells surrounded by 16 peripheral cells arranged in a shield (**D**). Note cuticular cap (*arrowhead*) detached from the secretory cell lateral walls. Positive Nile Blue A reaction in the subcuticular space (*pink droplet*) and head cells (*intense blue staining*) of *Clinopodium pulegium* (**E**). Secretion in the subcuticular space stained positively (*dark violet*) for terpenes with NADI reagent in *M. croatica* (**F**)

to dominant C1 trichomes, C2 trichomes were less abundant in all examined *in vitro* plants and occurred more frequently on the adaxial leaf side.

Morphoanatomy of glandular trichomes in Micromeria and Clinopodium spp. in vitro

In micropropagated plants of all examined species, peltate trichomes at different secretory stages were observed (**Fig. 5**). Immature glands with wrinkled surface (**Fig. 5A**), indicative of the close attachment of the cuticle to the upper secretory cells' walls are predominantly located at the leaf base and closer to the midrib. During gland maturation, the secretory material is gradually secreted from the head cells into developing subcuticular space, formed by detachment and elevation of the cuticle, where the secretion was shown to accumulate in many Lamiaceae species (Werker, 1993; Serrato-Valenti et al., 1997; Zuzarte et al., 2010). Therefore, mature glands appear balloon shaped, and their surface becomes smoother, displaying occasional droplets of secreted material (**Fig. 5B**). Peltate trichomes exhibited rather uniform morphology across examined species and consisted of a broad basal cell embedded in the epidermis, a short unicellular stalk with cutinized walls and a round multicellular secretory head (*ca*. 50-60 μm in diameter, at secretory stage), with a variable number (8-20) of secretory cells, depending on the species (**Fig. 5C-D**).

Different types of capitate trichomes, differing in stalk length and secretory head structure, have been described (Werker et al., 1985; Giuliani & Maleci Bini, 2008). *In vitro* grown plants of examined *Micromeria* and *Clinopodium* species displayed two types of capitate trichomes, differing in size and structure (**Fig. 3C**). Differences in the morphological characteristics of various capitate trichome types reflect different secretory processes within trichomes, and probably distinct functions (Ascensão et al., 1999). Type 1 capitate trichomes (C1) were positioned at an angle to the leaf surface (**Fig. 6**). They were composed of one basal epidermal cell, short unicellular stalk with cutinized lateral walls and unicellular ellipsoidal head of *ca*. 20-25 μm in size (**Fig. 6A**). No cuticle elevation was observed, and the secretory product accumulated within the secretory cell. As suggested by Fahn (1988), the thickenings of the cuticle on the lateral walls of the neck cell, functioning also as a short stalk, probably prevent the backflow of secreted products into mesophyll tissue. In this way, trichomes remain structurally (and biosynthetically) isolated from the rest of the leaf, and can therefore, unlike leaf, produce large quantities of secretory material that is often phytotoxic (Nielsen et al., 1991; Werker, 2000).

Type 2 capitate trichomes (C2) are positioned upright and composed of conical, elongated, and highly vacuolated basal cell, uni- to bicellular stalk, and unicellular, usually elongated secretory

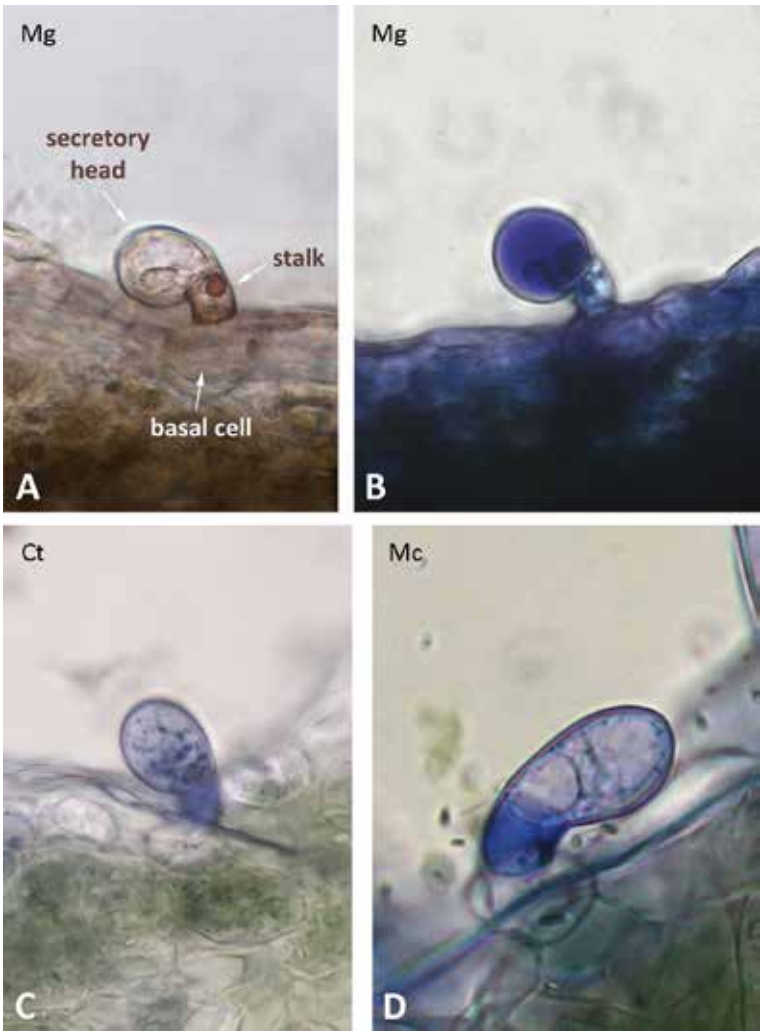

Fig. 6. Capitate trichomes type 1 on leaves of *in vitro* grown plants. **A-B** Longitudinal sections of C1 trichomes, showing large basal cell, short unicellular stalk and ellipsoidal unicellular head bent to the surface of *Micromeria graeca* leaf, after staining with Sudan IV (**A**) and Nile Blue A (**B**); **C-D** Longitudinal sections of C1 trichomes showing faint staining with NADI reagent of the secretion in the head cell of *Clinopodium thymifolium* (**C**) and *Micromeria croatica* (**D**). Note intense staining of the stalk cell lateral walls

head of *ca*. 10 µm in diameter (**Fig. 7**). Immature C2 trichomes (**Fig. 7A**, **Fig. 7D**) appear finger-like since elongated head cell is of the same diameter as the stalk cell(s). During maturation, secretory head of C2 trichomes develops spherical subcuticular space where the secretion accumulates (**Fig. 7B**, **Fig. 7E**, **Fig. 7F**), rendering trichome head rounded appearance (**Fig. 3C**). In the post-secretory phase, after cuticle rupture, subcuticular elevation is no longer visible, so that the glandular head of C2 trichomes no longer appears round but regains finger-like shape (**Fig. 7C**).

Type 2 capitate trichomes of *in vitro* grown plants correspond to those described by Werker et al. (1985) for the Lamiaceae, and are very similar to those detected in *Salvia officinalis* (Corsi & Bottega, 1999) or *Lavandula pedunculata* (Zuzarte et al., 2010). Nevertheless, Kremer et al. (2021) distinguished 4 subtypes of capitate trichomes in studied *Micromeria* and *Clinopodium* spp. Besides C1 capitate subtype, present in all investigated *Micromeria* and *Clinopodium* species, the authors

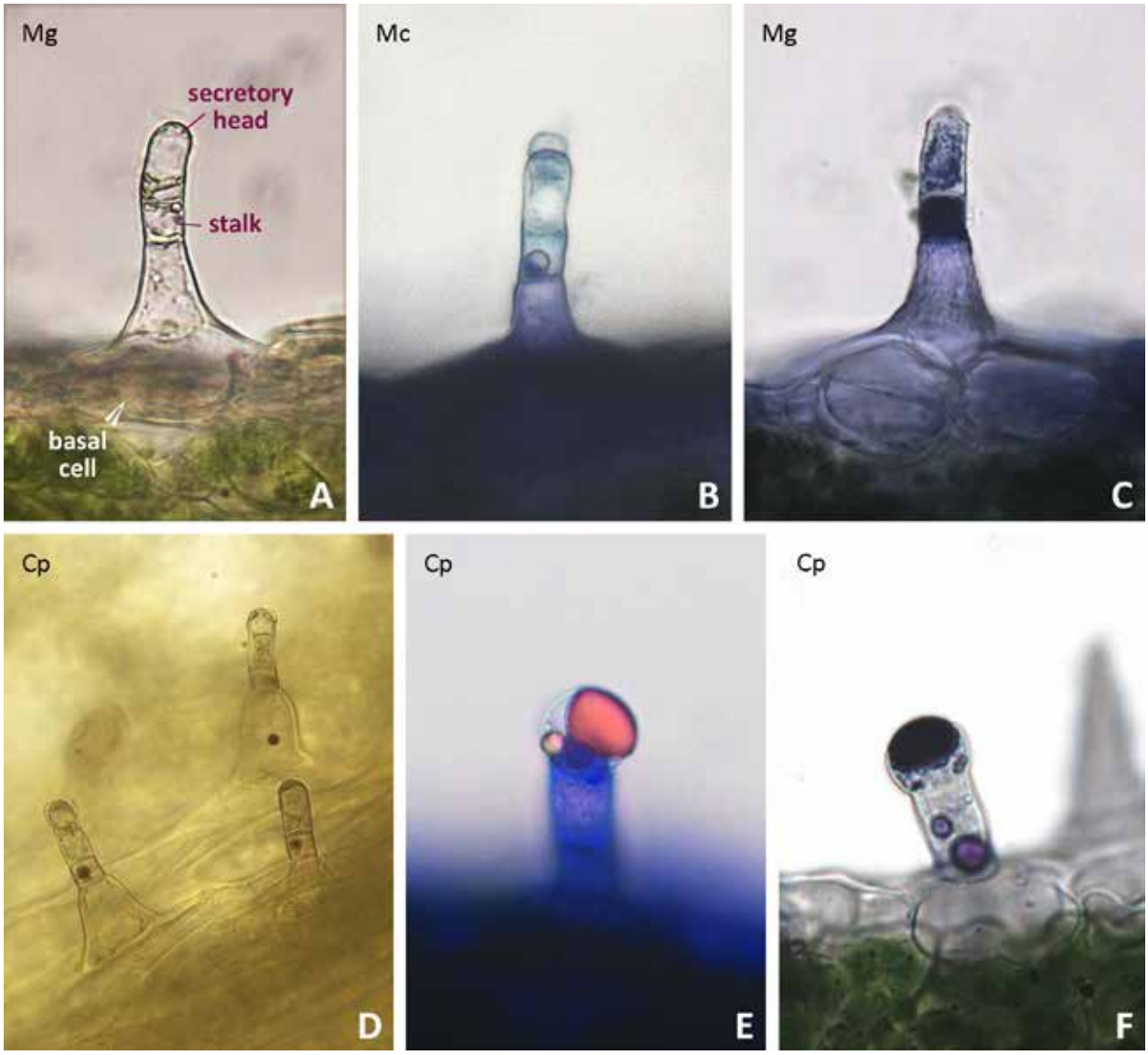

Fig. 7. Capitate trichomes type 2 on leaves of *in vitro* grown plants. Longitudinal sections of C2 trichomes, showing vacuolated pyramidal basal cell, subtending cylindrical unicellular stalk and unicellular head, both of approximately same diameters, after staining with Sudan IV (**A, D**), Nile Blue A (**B**, **E**) and NADI reagent (**C**, **F**). Note secretion accumulated in the subcuticular space of the early (**B**) and late secretory-stage trichomes (**E**, **F**), rich in essential oils (**B**, **E**) and terpenoids (**F**); in post secretory stage trichome (**C**), following rupture, the cuticle remains firmly attached to the cell wall in the basal part of the secretory cell, but no subcuticular elevation is visible. Mg – *Micromeria graeca*, Mc – *Micromeria croatica*, Cp –*Clinopodium thymifolium*

observed C2 subtype trichomes (a rounded head cell) only in *Micromeria* species studied, whereas roundish-head C3 and finger-like C4 (an elongated head cell, as narrow as the stalk cells, and only slightly enlarged above) subtypes were observed only in *Clinopodium* taxa. Based on these findings, and since the investigated taxa of the genus *Clinopodium* belong to the former *Micromeria* section *Pseudomelissa*, the authors concluded that micromorphological traits also support the recent transfer of *Micromeria* sect. *Pseudomelissa* to the genus *Clinopodium* (Kremer et al., 2021). However, these claims were based solely on scanning electron micrographs, which do not always allow precise determination of the cell number or shape. In our study, both SEM and light micrographs showed that the appearance of C2 trichomes differed depending on trichome developmental stage and secretory phase, as well as on sectioning plane. Capitate trichomes type 2 that we observed in both investigated *Micromeria* species fully corresponded to those observed in *Clinopodium* species. Finger-like capitate trichomes were observed in both taxa, and were indicative of early secretory-stage trichomes, with still developing subcuticular space. As the secretion continued, and more secretory material accumulated in this space, the morphology of the glandular head changed from roundish to spherical, as observed in *M. croatica* (**Fig. 3C**) as well as in *C. thymifolium* (**Fig. 3B**) and *C. pulegium* (**Fig. 7E**).

Histochemical characterization of secretions of glandular trichomes in Micromeria and Clinopodium spp. in vitro

Histochemical studies enable preliminary identification of the classes of bioactive compounds in tissues and cell compartments and are often employed to localize *in situ* the main classes of secondary metabolites present in plant secretions. Together with morphological and structural investigations, histochemical studies provide data on secretory modes and secretions, thus allowing us to postulate a functional role for different glandular trichome types in plant interactions with its abiotic and biotic environment.

The main classes of metabolites secreted by glandular trichomes of micropropagated plants were characterized by the histochemical tests in plant exudates *in situ* (**Figs. 5-7**). In all examined species, peltate trichome secretion contained lipophilic substances, as revealed by Sudan IV, and was for the most part composed of essential oil (**Tab. 1**). Nile Blue A staining for neutral and acidic lipids gave positive reaction in the subcuticular space and to a lesser degree in secretory cells and was particularly intense in *Clinopodium* species (**Fig. 5E**, **Tab. 1**). The presence of terpenoid compounds in this trichome morphotype was evidenced by intense dark-violet staining, with NADI reagent, of the secretory product within subcuticular space of all examined species (**Fig. 5F**). The use of NADI reagent demonstrated the presence of terpenoids (resiniferous acids and essential oils) in the trichome glandular cells or in their secretion in a number of plant species belonging to Lamiaceae (Corsi & Bottega, 1999; Ascensão et al., 1999; Marin et al., 2013).

In all examined species, capitate trichomes type 1 of *in vitro* grown plants stained weakly positive for lipophilic secretion (**Fig. 6**, **Tab. 1**). On the other hand, type 2 capitate trichomes exhibited much stronger histochemical reactions (**Fig. 7**, **Tab. 1**). Lipophilic secretory products accumulated in the subcuticular space of C2 trichomes, and small amounts could also be observed within secretory cells. That the majority of those lipophilic substances were the essential oils was shown by Nile Blue A staining (**Fig. 7B**, **E**). NADI reaction resulted in an intense dark-violet staining of the secretory product, thus confirming the presence of terpenoid compounds in type 2 capitate trichomes (**Fig. 7C**, **F**). Although it is generally assumed that the bulk of the essential oil in Lamiaceae is produced by peltate trichomes, studies showed that capitate trichomes in some species seem to produce significant amounts of essential oils (Ascensão et al., 1999; Mota et al., 2013; Stojičić et al., 2016).

Reports on the histochemical detection of different secondary metabolites carried out in plants

Table 1. Histochemical characterization of secretions of leaf glandular trichomes of selected *Micromeria* and *Clinopodium* species

Chemical compounds	*Micromeria graeca*			*Micromeria croatica*			*Clinopodium thymifolium*			*Clinopodium pulegium*		
	P	C1	C2	P	C1	C2	P	C1	C2	P	C1	C2
Lipids	++	±	+	++	±	+	++	+	±	+	+	++
Essential oils	+	±	+	+	±	+	++	+	±	++	+	++
Terpenoids	++	±	+	++	±	+	++	+	±	++	+	++

grown *in vitro* are scarce (Uzelac et al., 2015; Stojičić et al., 2016; Dantas et al., 2017; Tošić et al., 2019). Defined environment of *in vitro* culture provides plants with nearly optimal growth conditions, in contrast to marked variations of growth conditions of wild-growing plants, which may contribute to increased secondary metabolite production *in vitro* reported for a number of species (Fraternale et al., 2003; Affonso et al., 2009; Tošić et al., 2019). Shoot cultures of many medicinal and aromatic plants have been shown to accumulate secondary metabolites to a greater extent compared to natural plants (Bassolino et al., 2015; Makowczyńska et al., 2016). Since *in vitro* cultured plants are by definition considered as juvenile-stage plants (Croteau et al., 1981), the reported quali-quantitative differences in the volatile profile between wild-growing plants and those grown *in vitro* could be the consequence of different ontological stage of the plants.

Conclusions

In recent decades, considerable research has focused on understanding and exploiting glandular trichomes ability to secrete phytochemicals that might improve plant resistance to pests, modify trichome metabolism towards improving exudate levels and quality traits and allow commercial production of useful compounds. Plant cell, tissue and organ culture is a valuable method for the multiplication of selected genotypes and chemotypes, enabling efficient clonal propagation regardless of the season as well as quantitative and qualitative modifications in the production of plant secondary metabolites by different physical and chemical factors.

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