

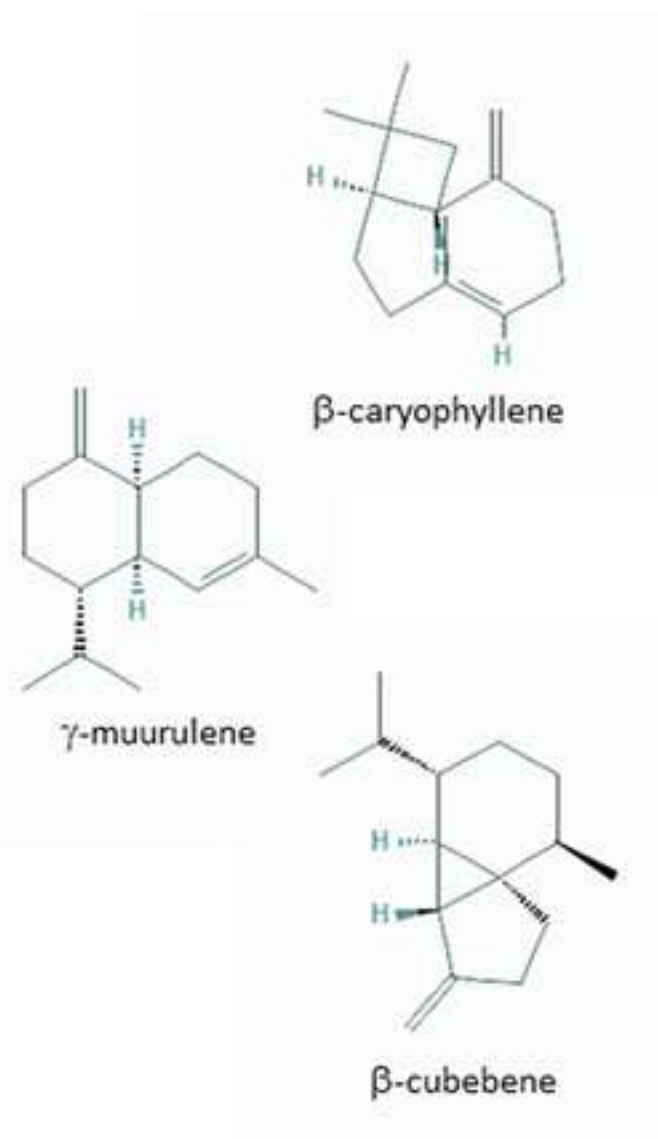
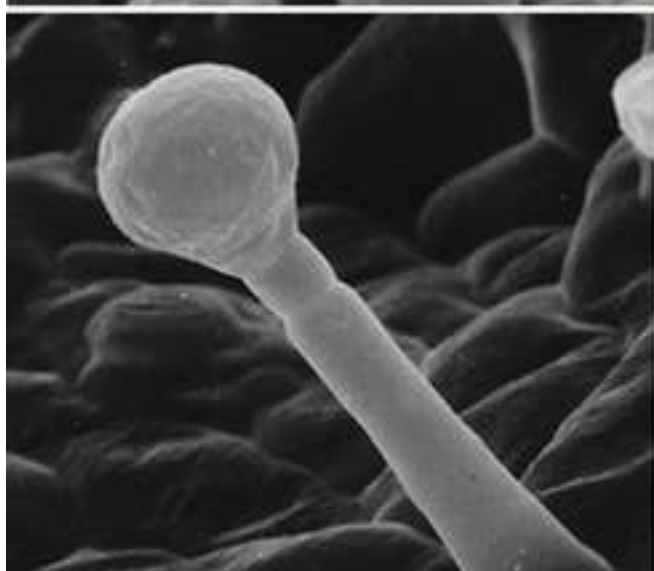
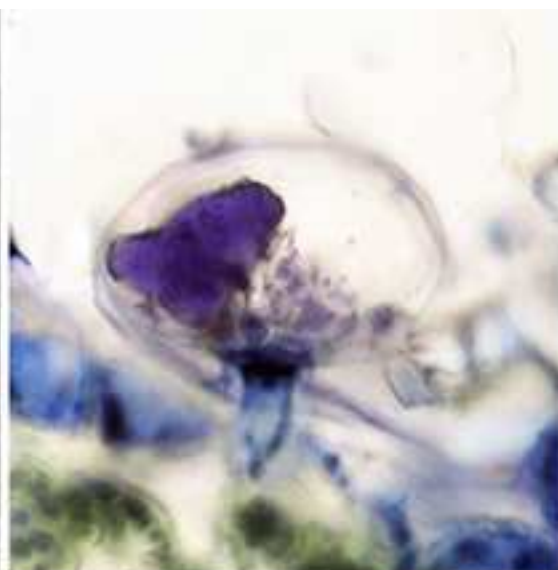
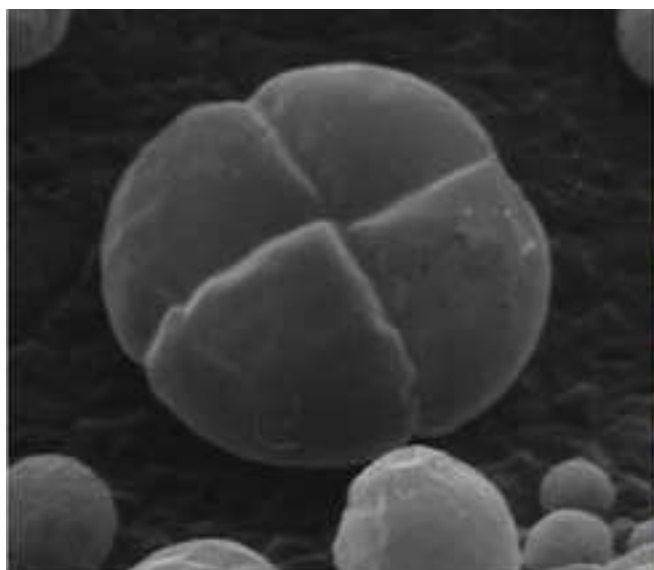
# Flora

## Morphology and phytochemistry of *Teucrium chamaedrys* L. (Lamiaceae) cultivated in Northern Italy --Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Wild germander, microscopy, trichomes, VOC, HS-SPME
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Abstract:	<p><i>Teucrium chamaedrys</i> L. (Lamiaceae) cultivated at the Ghirardi Botanic Garden (Lombardy, Northern Italy) was studied through a four-level research approach: 1) micromorphological and 2) histochemical to describe the features of the glandular trichomes on the vegetative and reproductive organs and the chemical nature of their secretory products, by means of light, fluorescence and scanning electron microscopy; 3) phytochemical, with the first characterization of the volatiles spontaneously emitted by leaves and flowers of samples of Italian origin by means of Head-Space Solid Phase Micro-Extraction (HS-SPME) coupled with Gas Chromatography–Mass Spectrometry (GC-MS); 4) ecological, through the combination of the morphological and phytochemical results with literature data concerning the ecology of the major volatile compounds.</p> <p>Three trichome morphotypes were observed, with a different distribution pattern on the vegetative and reproductive organs: peltates and short-stalked capitate, both ubiquitous on the whole plant, and long-stalked capitate, exclusive to the floral whorls. Both the peltates and the long-stalked capitate were recognized as the main sites of the terpene production. The HS-SPME analysis indicated that flowers displayed the most complex qualitative profile. Indeed, the vegetative and floral bouquets showed the predominance of sesquiterpene hydrocarbons and shared the main compounds, i.e., <math>\beta</math>-caryophyllene, <math>\gamma</math>-muurolene, and <math>\beta</math>-cubebene. From the literature data, a prevailing defensive action was highlighted both at the vegetative and reproductive organs level, along with the potential seductive role played by the floral bouquet.</p>
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## Highlights

- A multi-scale research approach was embraced for *Teucrium chamaedrys* L.
- Morphological, histochemical and phytochemical surveys were carried out
- The glandular *indumentum* was constituted by three trichome morphotypes
- Peltates and long-stalked capitates were responsible for terpene production
- The *bouquets* of volatiles spontaneously emitted by leaves and flowers were described



**Morphology and phytochemistry of *Teucrium chamaedrys* L. (Lamiaceae) cultivated in Northern Italy**

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31    **Abstract**

32    *Teucrium chamaedrys* L. (Lamiaceae) cultivated at the Ghirardi Botanic Garden (Lombardy,  
33    Northern Italy) was studied through a four-level research approach: 1) micromorphological  
34    and 2) histochemical to describe the features of the glandular trichomes on the vegetative and  
35    reproductive organs and the chemical nature of their secretory products, by means of light,  
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37    characterization of the volatiles spontaneously emitted by leaves and flowers of samples of  
38    Italian origin by means of Head-Space Solid Phase Micro-Extraction (HS-SPME) coupled with  
39    Gas Chromatography–Mass Spectrometry (GC-MS); 4) ecological, through the combination of  
40    the morphological and phytochemical results with literature data concerning the ecology of the  
41    major volatile compounds.

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43    vegetative and reproductive organs: peltates and short-stalked capitates, both ubiquitous on  
44    the whole plant, and long-stalked capitates, exclusive to the floral whorls. Both the peltates  
45    and the long-stalked capitates were recognized as the main sites of the terpene production.  
46    The HS-SPME analysis indicated that flowers displayed the most complex qualitative profile.  
47    Indeed, the vegetative and floral *bouquets* showed the predominance of sesquiterpene  
48    hydrocarbons and shared the main compounds, *i.e.*,  $\beta$ -caryophyllene,  $\gamma$ -muurolene, and  $\beta$ -  
49    cubebene. From the literature data, a prevailing defensive action was highlighted both at the  
50    vegetative and reproductive organs level, along with the potential seductive role played by the  
51    floral *bouquet*.

52

53    **Keywords**

54    Wild germander, microscopy, trichomes, VOC, HS-SPME

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## 1. Introduction

*Teucrium chamaedrys* L. (Lamiaceae), commonly called wild germander, is a suffruticose, highly polymorphic species native to Europe and South West Asia (Nencini et al., 2014). It presents a branched rhizome and numerous simple stems, which can reach up to 30 cm high, erect ascending, quadrangular, and hairy. The leaves are pubescent, oblong in shape with entire to incise-serrate or crenate-dentate margins and resemble miniature oak leaves (the specific epithet *chamaedrys* means "ground oak", referring to their shape and ground cover habit). The flowers are pale to deep purple in colour and appear in terminal clusters in late spring to early summer (Tutin and Wood, 1972). The species grows on arid meadows and rocky and sunny slopes, in xerophilous pine and oak woods from the sea level up to 1,700 m a.s.l. It is also ornamentally grown for its attractive, aromatic, and evergreen foliage. Since ancient times, the flowering aerial parts were used in traditional medicines in form of aqueous infusions or compresses for the treatment of digestive, respiratory, and gynaecological disorders. They were also used in case of wound healing and haemorrhoids, to stimulate diuresis and, in more recent times, to reduce body weight (Bosisio et al., 2004; Bulut et al., 2017; Güneş et al., 2017; Jarić et al., 2015; Matejić et al., 2020; Nencini et al., 2014). Regarding the taxonomy, this species belongs to *Teucrium* sect. *Chamaedrys* (Mill.) Schreb. and includes several subspecies distinguished by the leaf features, the general pubescence, and the ecological preferences (Pignatti et al., 2017-2019). Concerning the micromorphology, the literature offered different works regarding the characterization of the glandular and non-glandular *indumenta*. Two previous contributions on several species, including *T. chamaedrys* from Italy and Croatia, documented the existence of taxonomically valuable microcharacters related to the trichome morphotypes and distribution pattern on stems, leaves, and calices (Bini Maleci and Servettaz, 1991; Grubešić et al., 2007). The glandular *indumentum* was also examined on leaves and nutlets of different subspecies of *T. chamaedrys* from Turkey (Ecevit-Genç et al., 2017; Kaya et al., 2009), proving its taxonomic validity for the infraspecific treatment. As it pertains to the histochemical information on *T. chamaedrys*, a single work reported preliminary and general evidence regarding the chemical nature of the secretory products of different trichome types (Bini Maleci et al., 1987). Phytochemical investigations on the volatile components referred to two studies on the characterization of the Volatile Organic Compounds (VOCs) spontaneously emitted by leaves of Turkish specimens using the Direct Thermal Desorption (DTD) technique (Karimi et al., 2011; Özel et al., 2006). In addition, the volatile profiles of congeneric species of different origins, including *T. marum*, *T. orientale*, *T. polium*, and *T. flavum*, were characterized by means of Headspace Solid-Phase Microextraction (HS-SPME) analysis (Djabou et al., 2013a; Sagratini et al., 2012; Yildirmiş et al., 2017; Zouaoui et al., 2020). Furthermore, the composition of the essential oils from aerial parts of *T. chamaedrys* of different origins was analysed (Bezić et al.,

2011; Djabou et al., 2013b; Hajdari et al., 2020; Kaya et al., 2009; Sajjadi and Shookohinia, 2010). Polar extracts were also examined (Gafner et al., 2003; Frezza et al., 2018; Milutinović et al., 2019), often in association with their biological activities, including antimicrobial (Djabou et al., 2013b) and antiphytoviral (Bezić et al., 2011), along with cytotoxic and pro-apoptotic effects in colorectal cancer cells (Milutinović et al., 2019). Despite the wide traditional uses of *T. chamaedrys*, several works documented some risks of hepatotoxicity related to the content of furanoneocloredane diterpenes, especially teucrin A (Bosisio et al., 2004; Nencini et al., 2014).

In this context, the work presented herein was primarily addressed at combining, for the first time, micromorphological and phytochemical approaches of study on *T. chamaedrys* cultivated at the Ghirardi Botanic Garden (University of Milan, Toscolano Maderno, BS, Italy). Therefore, we performed subsequent and complementary research phases: 1) microscopic – to describe the morphology and the distribution pattern of glandular and non-glandular trichomes on the vegetative and reproductive organs by means of Light Microscopy and Scanning Electron Microscopy; 2) histochemical - to identify the main classes of secondary metabolites occurring in the secretory products; 3) phytochemical - to depict, as an element of novelty, the VOC emission profiles from leaves and flowers by means of HS-SPME analysis; 4) ecological – to draw a link between the volatile profiles and the potential plant-environment interactions through literature evaluation of the ecological role of the main compounds. Finally, this work is part of an ongoing project entitled “Botanic Garden, factory of molecules” aimed at enhancing the plant heritage of the Ghirardi Botanic Garden according to multi-scale research perspectives: microscopic, phytochemical, and bio-ecological.

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## 125       **2. Materials and Methods**

### 126           **2.1 Plant material**

127       *Teucrium chamaedrys* is cultivated at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Lombardy, Italy) of the Department of Pharmaceutical Sciences of the University of Milan. The samplings for the micromorphological and the phytochemical surveys were carried out concurrently in June 2020. Voucher specimens, labelled GBG2020/028 and GBG2020/029, were stored in the *Herbarium* of the Ghirardi Botanic Garden.

### 132           **2.2 Micromorphology**

133       The trichome structure, distribution pattern, and histochemistry on the vegetative and reproductive organs (stems, leaves, bracts, calyces, and corollas) were examined by means of Light Microscopy (LM) and Scanning Electron Microscopy (SEM). At least ten replicates per each plant part were studied to assess the variability of the micromorphological features. Referring to the trichome distribution, we qualitatively evaluated it using the following symbols: (-) missing, not observed in any of the replicates; (±) sporadic in no more than four

139 replicates; (+) present in all the replicates; (++) abundant in all the replicates with a  
140 distribution on the whole organ surface.

141

### 142 **2.2.1 Light Microscopy (LM) and Fluorescence Microscopy (FM)**

143 The epidermal surfaces of the examined organs were preliminarily observed by LM using hand-  
144 cut sections. Also, FAA-fixed samples, subsequently dehydrated with ethanol and embedded in  
145 Technovit/Historesin were analysed; they were sectioned with a microtome.

146 The following histochemical dyes were used: Toluidine Blue as a general dye (Beccari and  
147 Mazzi, 1966), Fluoral Yellow-088 for total lipids (Brundrett et al., 1991), Nile Red for neutral  
148 lipids (Greenspan et al., 1985), Nadi reagent for terpenes (David et al., 1964), PAS-reaction  
149 for total polysaccharides (Beccari and Mazzi, 1966), Ruthenium Red for acid polysaccharides  
150 (Jensen, 1962), Alcian Blue for mucopolysaccharides (Beccari and Mazzi, 1966), and Ferric  
151 Trichloride for polyphenols (Gahan, 1984). Control stains were simultaneously carried out.  
152 Observations were performed under a Leitz DM-RB Fluo optical microscope equipped with a  
153 Nikon® digital camera.

### 154 **2.2.2 Scanning Electron Microscopy (SEM)**

155 For SEM observations, small segments of stem, leaf, bract, calyx, and corolla were FAA-fixed  
156 for 4 days, dehydrated with acetone, critical-point dried, and carbon gold-coated. Observations  
157 were performed using a Philips® XL 20 SEM operating at 15 kV.

158

## 159 **2.3 Phytochemistry**

### 160 **2.3.1 Volatile Organic Compounds (VOCs)**

161 Three leaves and three flowers were cut and immediately inserted into separate glass vials of  
162 suitable volume for the analysis.

163 *HS-SPME Sample analysis* – The headspace sampling conditions were as reported in Ascrizzi et  
164 al., 2017 (Ascrizzi et al., 2017). For the headspace samplings, Supelco SPME (Solid Phase  
165 Micro-Extraction) devices, coated with polydimethylsiloxane (PDMS, 100 µm) were used; the  
166 same new fibre, preconditioned according to the manufacturer instructions, was employed for  
167 all analyses. After a 30 min equilibration, the fibre was exposed to sample the headspace for  
168 30 min. Both the equilibration and sampling times were experimentally determined to obtain  
169 an optimal adsorption of the volatiles, and to avoid both under- and over-saturation of the  
170 fibre and of the mass spectrometer ion trap. Once sampling was finished, the fibre was  
171 withdrawn into the needle and transferred to the injection port of the GC-MS system. Both the  
172 sampling and desorption conditions were identical for all the samples. Furthermore, blanks  
173 were performed before each first SPME extraction and randomly repeated during each series.



174 Quantitative comparisons of relative peak areas were performed between the same compounds  
175 in the different samples.

176 *GC-MS analysis* - Gas chromatography–electron impact mass spectrometry (GC–EI–MS)  
177 analyses were performed with a Varian CP-3800 gas chromatograph (Varian Inc., Walnut  
178 Creek, CA, USA) equipped with an Agilent DB-5 (Agilent Technologies Inc., Santa Clara, CA,  
179 USA) capillary column (30 m × 0.25 mm; film thickness 0.25 µm) and a Varian Saturn 2000  
180 ion trap mass detector (Varian Inc., Walnut Creek, CA, USA). Analytical conditions were as  
181 follows: injector and transfer line temperatures, 220 and 240 °C, respectively; oven  
182 temperature programmed to rise from 60 to 240 °C, at 3 °C min<sup>-1</sup>; carrier gas, helium at 1 ml  
183 min<sup>-1</sup>; splitless injection. The identification of constituents was based on a comparison of their  
184 retention times with those of authentic samples (when available), comparing their linear  
185 retention indices relative to a series of pure *n*-hydrocarbons (C5–C25). Computer matching  
186 was also used against commercial (NIST 14) and laboratory-developed library mass spectra  
187 built up from pure substances and components of commercial essential oils of known  
188 composition and MS literature data (Adams, 2007).

189

### 190 **3. Results**

#### 191 **3.1 Micromorphological survey**

##### 192 **3.1.1 Trichome morphotypes**

193 The vegetative and reproductive organs were characterized by a glandular *indumentum*  
194 composed of peltate and capitate trichomes (**Figure 1**).

195 The peltate hairs were composed of one basal epidermal cell, one neck cell, and four secretory  
196 cells surmounted by a wide subcuticular space (**Figure 1 a, b**).

197 The capitates were distinguished into two subtypes based on the stalk length and the head  
198 features. The short-stalked were consisted of one basal epidermal cell, one neck cell, and a  
199 one(two)-celled secretory head surrounded by a thin subcuticular space (**Figure 1 c, d**); these  
200 hairs may be variously sunken into the epidermis or protrude on the surface of the organ  
201 bearing them. The long-stalked hairs were made up of one basal cell, a 1(2)-celled stalk, and  
202 one globose secretory head cell (**Figure 1 e, f**).

203 Also, non-glandular trichomes were observed. They were simple, multicellular, and uniseriate  
204 with an acute apex; the cell diameter was progressively smaller moving from the base to the  
205 apex. The cuticle was generally smooth and sporadically characterized by *micropapillae*  
206 sometimes lacking on the basal cells.

207

##### 208 **3.1.2 Trichome distribution**

209 The trichomes distribution pattern is evidenced in **Table 1** and **Figure 2**. The leaves exhibited  
210 peltate, short capitate, and simple non-glandular hairs on both sides (**Figure 2 a, b**); the

211 peltates occurred only on the interveinal region of the laminas, while the short capitates were  
212 present mainly on the veinal system (**Figure 2 a, b**). Stems and bracts displayed the same  
213 types of hairs as the leaves, but in a lower density (**Figure 2 c**).  
214 On the examined floral whorls, the three trichomes morphotypes were ubiquitous. However,  
215 the short capitates were more abundant on calyces (**Figure 2 d**). On the corolla tube the  
216 peltates prevailed, whereas on lower lips long capitate and simple non-glandular trichomes  
217 were detected (**Figure 2 e, f**); the cells of these last hairs were much wider than those of the  
218 corresponding hairs occurring on the vegetative organs.

219

### 220 **3.1.3 Glandular trichome histochemistry**

221 The results of the histochemical survey are shown in **Table 2** and **Figure 3**. Peltate trichomes  
222 produced chemically heterogeneous substances with polysaccharidic, flavonoid, and terpenic  
223 components (**Figure 3 a-d**). The short and long capitates produced exclusively a hydrophilic  
224 (mucopolysaccharides) and a terpenic secretion, respectively (**Figure 3 e, f**).

225

## 226 **3.2 Phytochemical survey**

### 227 **3.2.1 VOC profile**

228 The HS-SPME analysis revealed a total of 59 different compounds. Specifically, 39 and 45  
229 compounds were identified in the leaf and flower profiles, respectively (Table 3).  
230 Sesquiterpene hydrocarbons dominated the leaf profile (93.5%), followed by monoterpene  
231 hydrocarbons (2.6%). Oxygenated sesquiterpenes and non-terpene derivatives were present  
232 in comparable amounts (1.5%, 1.2%), while oxygenated monoterpenes accounted for 0.9%.  
233  $\beta$ -Caryophyllene (34, 27.1%) was the main compound, followed by  $\gamma$ -muurolene (42, 22.8%)  
234 and  $\beta$ -cubebene (31, 12.3%). (E)- $\beta$ -Farnesene (40, 6.6%),  $\alpha$ -cubebene (28, 5.5%),  $\beta$ -  
235 bourbonene (30, 5.2%), and  $\alpha$ -humulene (39, 3.8%) showed significant relative abundances,  
236 while (Z)- $\gamma$ -bisabolene (51), bicyclogermacrene (44),  $\beta$ -copaene (35),  $\beta$ -pinene (2),  $\alpha$ -  
237 gurjunene (33),  $\delta$ -cadinene (52), and dendrolasin (55) all exhibited relative concentrations in  
238 the range 2.0%-1.0%. The remaining compounds had relative abundances <1.0%, or were  
239 present in traces (<0.1%). Fourteen exclusive compounds were identified, all accounting for  
240 amounts lower than 1.0% or occurring in traces, of which the most abundant was  $\alpha$ -neo-  
241 clovene (38, 0.5%).

242 The flower profile was dominated by sesquiterpene hydrocarbons (83.2%), followed by  
243 oxygenated monoterpenes (9.1%) and non-terpene derivatives (4.5%). Monoterpene  
244 hydrocarbons (2.9%) and oxygenated sesquiterpenes (0.1%) were the least represented  
245 classes.  $\beta$ -Caryophyllene (34, 38.5) was the most abundant compound, followed by  $\gamma$ -  
246 muurolene (42, 12.0%) and  $\beta$ -cubebene (31, 11.2%). 1,8-Cineole (6, 6.7%),  $\alpha$ -humulene (39,  
247 6.1%),  $\alpha$ -cubebene (28, 4.3%),  $\alpha$ -copaene (29, 3.2%), and  $\beta$ -pinene (2, 2.4%) showed

248 remarkable abundances, while phenylethyl alcohol (16), 3-octanone (3), (E)- $\beta$ -farnesene (40),  
249  $\beta$ -bourbonene (30), and methyl carvacrol (23) all ranged between 2.0% and 1.0%. The  
250 remaining compounds showed percentages <1.0% or were detected in traces. Twenty exclusive  
251 compounds were detected, among which  $\alpha$ -copaene (29, 3.2%) dominated, followed by  
252 phenylethyl alcohol (16, 1.6%), 3-octanone (3, 1.3%), and methyl carvacrol (23, 1.1%); the  
253 other exclusive compounds were identified with abundances <1.0% or in traces.  
254 Leaf and flower profiles shared 25 common compounds, comprising the 3 dominant ones, i.e.,  
255  $\beta$ -caryophyllene (34),  $\gamma$ -muurolene (42), and  $\beta$ -cubebene (31). Moreover, 8 compounds were  
256 more abundant in the leaves (28, 30, 33, 35, 40, 44, 51, 52), 3 in the flowers (2, 6, 39), while  
257 the others showed comparable amounts, lower than 1.0%.

258

#### 259 **4. Discussion**

260 The micromorphological investigations on *T. chamaedrys* showed the presence of glandular  
261 trichomes, i.e., peltate and capitate, typical of the Lamiaceae family (Giuliani and Maleci Bini,  
262 2008). The structure of both morphotypes was consistent with the results of previous works  
263 carried out on the vegetative and reproductive organs. The peltate trichomes showed a 4-  
264 celled secreting head (Bini Maleci and Servettaz, 1991; Grubešić et al., 2007; Kaya et al.,  
265 2009); the short- and long-stalked capitates possessed a bicellular and monocellular head,  
266 respectively, matching the morphotypes known in literature: capitates of types I and II in Kaya  
267 et al. (2009), different subforms of types B capitates in Grubešić et al. (2007), capitates of  
268 type B and C in Bini Maleci and Servettaz (1991), sessile and clavate trichomes in Ecevit-  
269 Genc et al. (2017).

270 Additionally, the simple, uniseriate, non-glandular trichomes observed herein were detected in  
271 previous studies, and defined as aciculate hairs (Bini Maleci and Servettaz, 1991; Grubešić et  
272 al., 2007; Kaya et al., 2009). The sporadic presence of cuticular ornamentations in the form of  
273 *micropapillae* was also confirmed. Nevertheless, coating flagelliform trichomes were detected  
274 on stems by Grubešić et al. (2007) and flask-shaped trichomes were observed on the margins  
275 of the calyx teeth by Bini Maleci and Servettaz (1991); both morphotypes were not detected  
276 herein. These differences could be related to the high level of infraspecific variability  
277 recognized within the target species.

278 The distribution pattern of the different trichomes morphotypes proved consistent with  
279 literature information, in particular with reference to the exclusive occurrence of the long-  
280 stalked capitates on the reproductive organs, with special reference to the calyx (Bini Maleci  
281 and Servettaz, 1991; Grubešić et al., 2007; Kaya et al., 2009). The short-stalked capitates  
282 were an exception since they resulted sporadic on the corollas, whereas previous works  
283 indicated them as lacking on this floral whorl (Bini Maleci and Servettaz, 1991).

284 Concerning the histochemical survey, this work represents the first contribution in which the  
285 chemical nature of the secretory products was documented using digital light microscopy

techniques. Bini Maleci et al. (1987) reported only generic information regarding the heterogeneous secretion with a low lipid content for the peltates, and the exclusive hydrophilic and lipophilic secretions for the short- and the long-stalked capitate, respectively. This evidence matched the results of our survey. In fact, in the peltates the synthesis of a complex mixture of secretory products was confirmed, due to the co-occurrence of terpenic, polysaccharidic, and polyphenolic fractions. This heterogeneous secretion was also highlighted in the peltates of other Lamiaceae species, e.g. *Scutellaria brevibracteata* (Giuliani et al., 2020c), whereas in *S. altissima*, *S. caucasica*, *Lavandula dentata*, and *Ballota acetabulosa* these trichomes turned out to be terpene producers exclusively (Giuliani et al., 2021, 2020d, 2020a, 2020b). Concerning the short-stalked capitate, the muco-polysaccharidic secretion was widely documented in most of the studied members of the Lamiaceae family (Giuliani and Maleci Bini, 2008). The long-stalked capitate were exclusive terpene factories in *T. chamaedrys*, whereas they produced a complex secretion in *S. brevibracteata*, *S. altissima*, *S. caucasica*, and *B. acetabulosa* (Giuliani et al., 2021, 2020c, 2020d, 2020a). The histochemical results, coupled with the evaluation of the trichome distribution pattern, allowed us to correlate the different morphotypes with the volatile emission profiles from leaves and flowers. Indeed, we can argue that the long capitate represent the main sites for the terpene secretion at floral level; in addition, peltates, widespread on all the epidermal surfaces, also contribute, through a synergistic action, to the production of volatiles on the reproductive organs. At the stem and leaf level, the terpene production and emission are exclusively related to the activity of the peltates. In this work, we emphasized the well-known importance of calyx characters as distinctive taxonomical hints within *Teucrium*, as already established for other Lamiaceae genera, e.g., *Stachys* and *Salvia* (Giuliani and Maleci Bini, 2008). Indeed, plant trichomes are still of great interest to descriptive and experimental botanists and data on *indumenta* are routinely considered in modern taxonomic investigations. As simple morphological tools, trichomes are helpful due to their broad occurrence on plant surfaces and how easily they can be examined. In addition, comparative morphological data may be valuable for evolutionary studies, and for the physio-ecological roles they played. Concerning the phytochemistry, the HS-SPME analysis highlighted a more complex qualitative profile in flowers, due to the highest number of total and exclusive compounds. Nevertheless, both compositions were dominated by the sesquiterpene hydrocarbons and shared the most abundant compounds:  $\beta$ -caryophyllene (34),  $\gamma$ -muurolene (42), and  $\beta$ -cubebene (31). The exclusive compounds did not allow to detect marked differences between the *bouquets*, since they showed very low relative abundances (<1.0%) in leaves, and in flowers only  $\alpha$ -copaene (29) occurred with an appreciable amount (3.2%). Comparison with previous studies focusing on VOCs of the target species was difficult, due to the different analytical methods employed (Karimi et al., 2011; Özel et al., 2006). However, sesquiterpene hydrocarbons were confirmed as the dominant chemical class and  $\beta$ -

325 caryophyllene as the major constituent; the other main molecules were, instead, the alpha-  
 326 isomeric forms of muurolene and cubebene (Karimi et al., 2011; Özel et al., 2006), recorded in  
 327 our samples with relative percentages equal to 0.4% (46, leaves) and 5.5/4.3% (28,  
 328 leaves/flowers), respectively.

329 The other major compounds did not match those of the Turkish species, for which  $\beta$ -pinene,  
 330 germacrene D,  $\alpha$ -pinene,  $\alpha$ -farnesene,  $\alpha$ -gurjunene,  $\gamma$ -elemene, and  $\gamma$ -cadinene were recorded  
 331 (Özel et al., 2006). Indeed, in the profiles analysed herein, germacrene D,  $\alpha$ -farnesene, and  $\gamma$ -  
 332 elemene were absent, whereas the remaining compounds showed relative amounts <3.0% (1,  
 333 2, 33, 50).

334 Similar considerations arose for the previously examined congeneric species. Indeed, although  
 335 the sesquiterpene hydrocarbons invariably constituted the dominant chemical family, a high  
 336 level of variability emerged among the most abundant compounds. Only  $\beta$ -caryophyllene  
 337 resulted common to our samples, *T. marum*, *T. flavum* and *T. polium* (Djabou et al., 2013a;  
 338 Sagratini et al., 2012; Yildirmiş et al., 2017; Zouaoui et al., 2020). Concerning the other two  
 339 main compounds of our samples, muurolene and cubebene occurred as alpha isomers in the  
 340 Algerian species with relative abundances <2.0% (Zouaoui et al., 2020), while they were  
 341 absent in the other literature profiles.

342 Other phytochemical works referred to the essential composition analysis of samples of  
 343 different origins (Bezić et al., 2011; Djabou et al., 2013b; Hajdari et al., 2020; Kaya et al.,  
 344 2009; Sajjadi and Shookohinia, 2010). Sesquiterpene hydrocarbons invariably prevailed in all  
 345 the profiles, despite the differences related to the analysed plant parts, the geographical  
 346 provenience and the analytical technique.  $\beta$ -Caryophyllene (34) was confirmed as dominant for  
 347 the Croatian, Iranian, and Turkish samples (Bezić et al., 2011; Kaya et al., 2009; Sajjadi and  
 348 Shookohinia, 2010), while germacrene D prevailed in the species from Macedonia (Hajdari et  
 349 al., 2020). Additionally, germacrene D was the second most abundant compound in all the  
 350 profiles from the literature. On the contrary,  $\gamma$ -muurolene (42) was not detected in any of the  
 351 other profiles and  $\beta$ -cubebene (31) was found only in the Turkish subspecies (*T. chamaedrys*  
 352 subsp. *trapezunticum* 3.2%; *T. chamaedrys* subsp. *sypsiense* 0.7%) (Kaya et al., 2009).

353 Regarding the ecological role of the volatilome, the prevalence of sesquiterpene hydrocarbons  
 354 in both the vegetative and floral *bouquets* suggested prevailing defensive mechanisms, as  
 355 widely recognized to this chemical family (Giuliani et al., 2020d). With regards to the dominant  
 356 compounds,  $\beta$ -caryophyllene (34) could develop a repellent action towards adult individuals of  
 357 pests such as *Tribolium castaneum*, *Lasioderma serricorne*, and *Liposcelis bostrychophila* (Cao  
 358 et al., 2018), as well as parasites and herbivores (Giuliani et al., 2020c). Specifically, as an  
 359 example, this molecule could be active in plant-herbivore tritrophic interactions, recalling  
 360 nematodes able to kill parasites such as *Diabrotica virgifera* (Degenhardt et al., 2009). In  
 361 addition,  $\beta$ -caryophyllene (34) and  $\beta$ -cubebene (31) were detected among the main  
 362 compounds of essential oils with moderate insecticidal activity against *L. serricorne* and *T.*  
 363 *castaneum* (Wang et al., 2015). Antifeedant and nematocidal properties were documented for

364 essential oils added with  $\beta$ -cubebene (31) (Cao et al., 2018; Sosa et al., 2012). This defensive  
 365 action could be enhanced by  $\gamma$ -muurolene (42) (Chizzola, 2013; Giuliani et al., 2020d) and  
 366 1,8-cineole (6). As a matter of fact, acaricidal, fumigant, and larvicidal properties were  
 367 attributed to the latter compound (Hu et al., 2015; Lucia et al., 2012), as well as antifungal  
 368 and anti-ochratoxigenic activity against *Aspergillus carbonarius* (Dammak et al., 2019). In  
 369 addition, 1,8-cineole (6), in synergy with  $\beta$ -pinene (2), could play a crucial role in allelopathy  
 370 (Nishida et al., 2005). A defensive action could also be attributed to the most abundant  
 371 exclusive floral compound,  $\alpha$ -copaene (29), considering its potential larvicidal property against  
 372 *Culex quinquefasciatus* (Senthilkumar et al., 2008). Nevertheless, literature information on this  
 373 compound also revealed an attractive capability towards ambrosia beetles, vectors of pathogen  
 374 fungi. Although this action seems to conflict with a defensive role, the emission of such a  
 375 compound could be a "signal" to detect the presence of the pest and, in turn, prevent the  
 376 disease onset (Kendra et al., 2016; Owens et al., 2019). Moreover, other defensive  
 377 mechanisms could be expressed through the emission of (*E*)- $\beta$ -farnesene (40), which  
 378 possesses larvicidal effects against mosquitos of the genera *Anopheles*, *Aedes*, and *Culex*  
 379 (Govindarajan and Benelli, 2016), along with a control role towards aphids (Yu et al., 2012).  
 380 However, the main compounds were also shown to be involved in seductive strategies.  $\beta$ -  
 381 Caryophyllene (34) could be involved in the preselection and attraction of pollinators (Giuliani  
 382 et al., 2020c; Novaković et al., 2019). Specifically, its emission from flowers could stimulate  
 383 positive feedback in honeybees as *Apis cerana* (Abraham et al., 2018) and *A. mellifera*  
 384 (Giuliani et al., 2020e), as well as in non-pollinators as *Vespa velutina* and *Bombus* spp.  
 385 (Giuliani et al., 2021; Zhang, 2018). In addition, the high amount of  $\gamma$ -muurolene (42) during  
 386 the flowering stage could be related to the pollination period and to a simultaneous protective  
 387 action against microorganisms, diseases, and herbivores (Fernandez et al., 2021). Attractive  
 388 roles towards pollinators were also known for  $\alpha$ -humulene (39),  $\beta$ -pinene (2), and 1,8-cineole  
 389 (6), often in association with  $\beta$ -caryophyllene (34) (Abraham et al., 2018; Giuliani et al.,  
 390 2020b, 2020a; Stevenson, 2019; Zhang, 2018). Finally,  $\beta$ -bourbonene (30) was documented  
 391 as a chemical cue that can drive the foraging preferences of bees (Cellini et al., 2019), while  
 392 (*E*)- $\beta$ -farnesene (40), abundant in the floral *bouquet* of *Orchis pauciflora*, could contribute to  
 393 the increase of the foraging rate of pollen grains by *Bombus terrestris* (Valterová et al., 2007).  
 394 In this context, the sharing of the main compounds by the two profiles did not allow to clearly  
 395 differentiate the potential ecological roles between the vegetative and the floral *bouquets*.  
 396 However, some hypotheses emerged by evaluating the relative abundance of each molecule.  
 397 The dual role of  $\beta$ -caryophyllene (34) and  $\gamma$ -muurolene (42) suggests an attractive role at the  
 398 flower level and a defensive action at the leaf level, enhanced by  $\beta$ -cubebene (31). Similar  
 399 considerations emerged for 1,8-cineole (6) and  $\beta$ -pinene (2), whose higher relative amounts at  
 400 floral level may be related to a prevalent attractive role; on the other hand, (*E*)- $\beta$ -farnesene  
 401 (40), more abundant in leaves, could be related to a predominant defensive action. The  
 402 attractive role of  $\alpha$ -humulene (39) and  $\beta$ -bourbonene (30) could be equally distributed, since



403 the former is more abundant in flowers, while the latter in leaves. Finally, the defensive  
404 property of  $\alpha$ -copaene (29), exclusive to the flowers but not significantly abundant, could be  
405 associated with a “warning signal” to prevent the attack by potential parasites.

406

## 407 **5. Conclusions**

408 The multidisciplinary approach of this investigation allowed us to depict the target species by  
409 combining the morphological characterization of the glandular *indumentum* with the production  
410 of volatile molecules.

411 Three trichome morphotypes were detected, each with a peculiar distribution pattern on the  
412 vegetative and reproductive components. We documented, as an element of novelty, the  
413 histochemical nature of the secretory products by means of digital light microscopy techniques.  
414 The peltate and long-stalked capitate morphotypes were the main sites where the secretion  
415 and release of terpenes occurs. In addition, for the first time, the VOC emission profile of  
416 plants cultivated in Italy was characterized through HS-SPME, with  $\beta$ -caryophyllene,  $\gamma$ -  
417 muurolene, and  $\beta$ -cubebene as dominant compounds.

418 The correlation of the phytochemical results with the ecological role ascribed by the literature  
419 to the major compounds allowed us to hypothesize a prevailing defensive role for the  
420 vegetative and floral *bouquets*, with a co-occurring potential attractive action at flower level.  
421 Finally, this work enriched knowledge in the micromorphological, phytochemical, and bio-  
422 ecological fields of the plant heritage preserved at the Ghirardi Botanic Garden, as part of the  
423 project “Botanic Garden, factory of molecules”.

424

## 425 **Funding**

426 The authors are grateful to the Lombardy Region for the financial support of the project  
427 “Botanic Garden, factory of molecules”, under the Call for the Enhancement of Museums Lr.  
428 25/2016, year 2019.

429

## 430 **Conflicts of interest**

431 The authors declare no conflict of interests.

432

## 433 **Acknowledgements**

434 We would like to thank Mr. Leonardo Molino for his help in the English revision. We are also  
435 grateful to Mr. Mauro Folli and Mr. Giacomo Folli, gardeners at the Ghirardi Botanic Garden, for  
436 their precious work in the care of the cultivated plants.

437

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636 **Table 1.** Distribution pattern of the glandular and non-glandular trichomes in *Teucrium*  
 637 *chamaedrys* L.

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Trichome type	Stem	Leaf		Bract		Calyx		Corolla	
		adax	abax	adax	abax	adax	abax	adax	abax
<b>peltate</b>	±	+	++	+	+	-	+	-	++
<b>short capitate</b>	+	+	+	±	+	-	+	-	±
<b>long capitate</b>	-	-	-	-	-	-	+	-	++
<b>simple non-glandular</b>	+	+	+	+	±	+	+	+	+

639 Symbols: (-) missing, (±) sporadic, (+) present, (++) abundant

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660 **Table 2.** Results of the histochemical tests on the glandular trichomes of *Teucrium*  
661 *chamaedrys* L.

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Stainings	Target-compounds	peltate	short capitate	long capitate
<b>Fluoral Yellow-088</b>	Total lipids	±	–	+
<b>Nile Red</b>	Neutral lipids	±	–	+
<b>Nadi reagent</b>	Terpenoids	+	–	++
<b>Ruthenium Red</b>	Acid polysaccharides	+	+	–
<b>Alcian Blue</b>	Muco-polysaccharides	+	±	–
<b>Ferric Trichloride</b>	Polyphenols	++	–	–

663 Symbols: (–) negative response; (±) sporadically positive response; (+) positive response; (++) intensely positive response

683 **Table 3.** HS-SPME profile of leaves and flowers of *Teucrium chamaedrys* L.

	I.r.i.	Compounds	Relative Abundance (%)	
			Leaves	Flowers
1	941	$\alpha$ -pinene	0.9	0.5
2	982	$\beta$ -pinene	1.4	2.4
3	987	3-octanone	-	1.3
4	1009	(Z)-3-hexenol acetate	0.3	-
5	1032	limonene	0.2	tr
6	1034	1,8-cineole	0.7	6.7
7	1052	(E)- $\beta$ -ocimene	tr	-
8	1060	isopentyl butyrate	-	0.1
9	1070	cis-sabinene hydrate	-	tr
10	1071	1-octanol	-	0.3
11	1076	trans-linalool oxide (furanoid)	-	tr
12	1099	isopentyl-2-methyl butanoate	-	0.2
13	1101	linalool	-	0.2
14	1102	nonanal	tr	-
15	1105	isopentyl isovalerate	-	0.2
16	1110	phenylethyl alcohol	-	1.6
17	1140	nopinone	-	tr
18	1143	camphor	0.1	0.8
19	1178	4-terpineol	-	0.1
20	1199	n-dodecane	-	tr
21	1204	decanal	0.1	0.2
22	1240	(Z)-3-hexenyl isovalerate	0.1	-
23	1241	methyl carvacrol	-	1.1
24	1244	carvone	tr	-
25	1259	linalool acetate	-	0.2
26	1299	n-tridecane	-	0.5
27	1340	$\delta$ -elemene	0.3	0.2
28	1351	$\alpha$ -cubebene	5.5	4.3
29	1376	$\alpha$ -copaene	-	3.2
30	1384	$\beta$ -bourbonene	5.2	1.1
31	1390	$\beta$ -cubebene	12.3	11.2
32	1399	n-tetradecane	0.3	0.2
33	1410	$\alpha$ -gurjunene	1.1	0.5
34	1420	$\beta$ -caryophyllene	27.1	38.5
35	1429	$\beta$ -copaene	1.5	0.7
36	1439	$\alpha$ -guaiene	-	0.4
37	1441	aromadendrene	0.3	0.1
38	1454	$\alpha$ -neo-clovene	0.5	-
39	$\beta$	$\alpha$ -humulene	3.8	6.1
40	1460	(E)- $\beta$ -farnesene	6.6	1.2
41	1462	cis-muurolo-4(14),5-diene	0.7	0.4
42	1477	$\gamma$ -muurolene	22.8	12.0
43	1491	trans-muurolo-4(14),5-diene	0.4	-
44	1495	bicyclogermacrene	1.6	1.0
45	1497	epizonarene	-	0.3
46	1498	$\alpha$ -muurolene	0.4	-
47	1499	trans- $\beta$ -guaiene	-	0.4
48	1505	$\delta$ -amorphene	0.1	-
49	1507	(E,E)- $\alpha$ -farnesene	0.2	0.2



50	1513	<i>trans</i> - $\gamma$ -cadinene	tr	0.3
51	1515	( <i>Z</i> )- $\gamma$ -bisabolene	1.8	0.3
52	1524	$\delta$ -cadinene	1.1	0.6
53	1534	cadina-1,4-diene	0.3	0.2
54	1538	$\alpha$ -cadinene	0.2	-
55	1574	dendrolasin	1.0	-
56	1575	germacrene D-4-ol	0.3	-
57	1581	caryophyllene oxide	-	0.1
58	1600	<i>n</i> -hexadecane	0.4	-
59	1640	<i>epi</i> - $\alpha$ -cadinol	0.1	-
<b>Monoterpene hydrocarbons</b>			2.6	2.9
<b>Oxygenated monoterpenes</b>			0.9	9.1
<b>Sesquiterpene hydrocarbons</b>			93.5	83.2
<b>Oxygenated sesquiterpenes</b>			1.5	0.1
<b>Non-terpene derivatives</b>			1.2	4.5
<b>Total</b>			99.6%	99.8%

*l.r.i.* - Linear retention index (DB-5 column).

*tr* - compound detected in traces (<0.1%)

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705 **Captions to Figures**

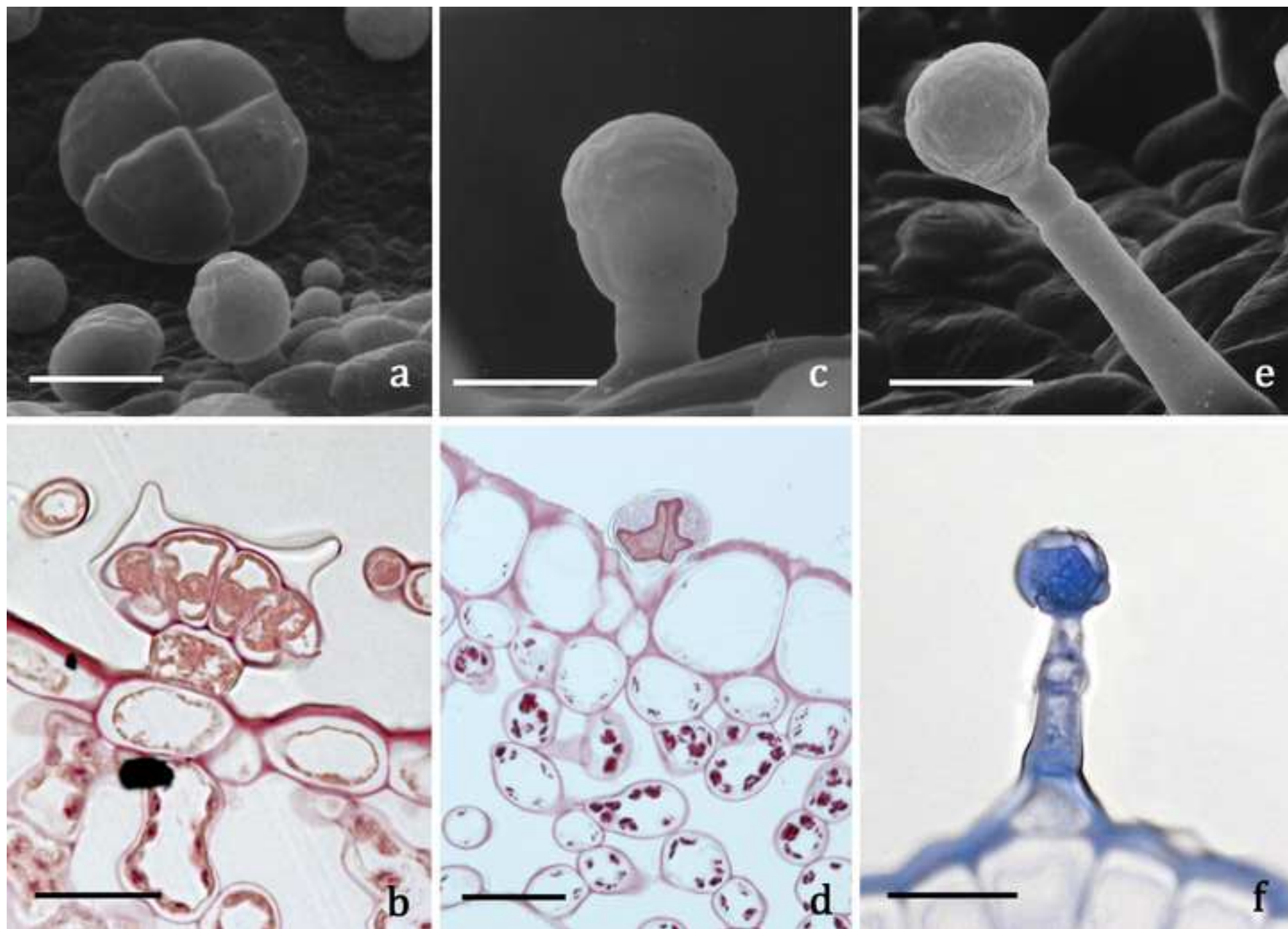
706 **Figure 1.** Trichome morphotypes in *Teucrium chamaedrys* L. **(a, b)** peltate trichome; **(c, d)**  
707 short-stalked capitate trichome; **(e, f)** long-stalked capitate trichome. **(a, c, e)** SEM  
708 micrographs; **(b, d, f)** LM micrographs, d-e, PAS reaction, f, Toluidine Blue. *Scale bars: a, d =*  
709 *25  $\mu\text{m}$ ; b, c, e, f = 20  $\mu\text{m}$ .*

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711 **Figure 2.** SEM micrographs showing trichome distribution pattern on the examined vegetative  
712 and reproductive organs of *Teucrium chamaedrys* L. **(a, b)** Leaf adaxial (a) and abaxial (b)  
713 surfaces with peltate, short capitates, and simple non-glandular trichomes. **(c)** Bract abaxial  
714 surface with peltates, short capitates, and simple non-glandular trichomes. **(d)** Calyx teeth,  
715 abaxial surface, with short capitates and simple non-glandular trichomes. **(e, f)** Corolla abaxial  
716 surface at the apical region with abundant long capitates. *Scale bars: a-f = 50  $\mu\text{m}$ .*  
717 *Symbols: peltate (asterisk); short-stalked capitate (arrowhead).*

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719 **Figure 3.** LM micrographs showing the histochemistry of the glandular trichomes in *Teucrium*  
720 *chamaedrys* L. **(a-d)** peltate: Alcian Blue (a), Aluminium trichloride (b), Nadi reagent (c),  
721 Fluoral yellow-088 (d). **(e)** Short-stalked capitate: Alcian Blue. **(f)** Long-stalked capitate: Nadi  
722 reagent. *Scale bars: a-c = 25  $\mu\text{m}$ ; d = 50  $\mu\text{m}$ ; e-f = 10  $\mu\text{m}$ .*

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Figure 1



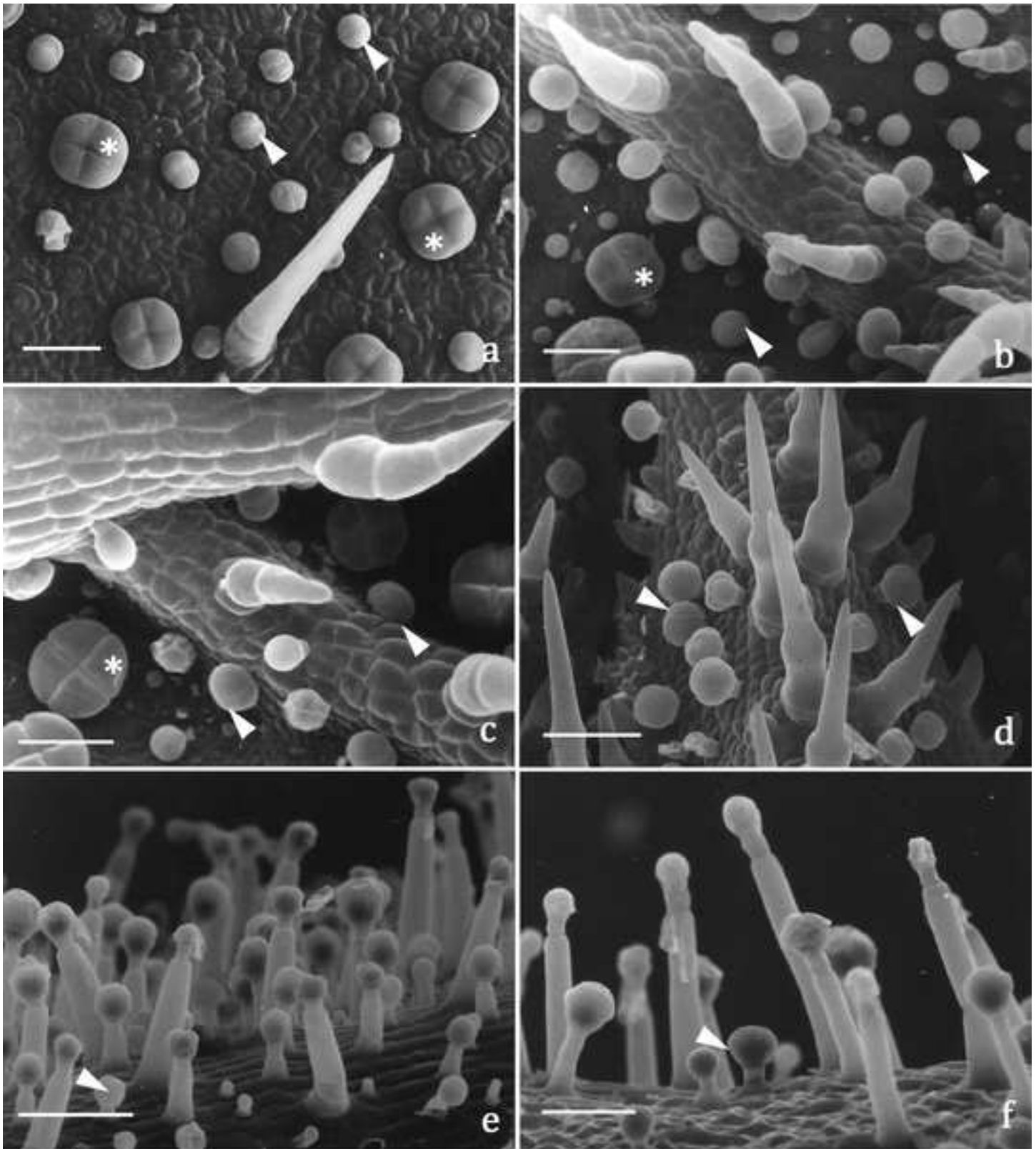
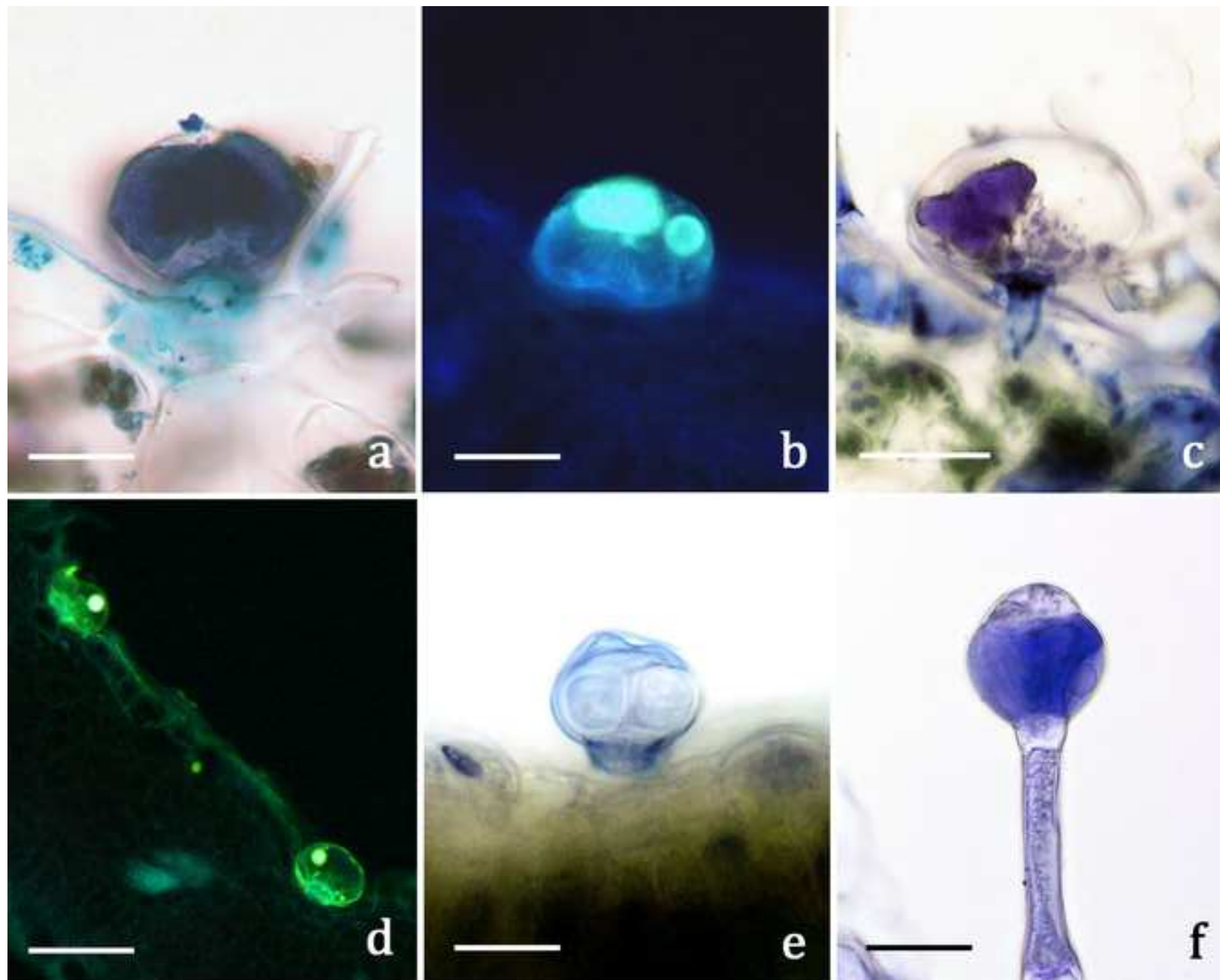


Figure 3



### Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Milan, 20th April 2021

For all the authors,

Claudia Giuliani

A handwritten signature in black ink, appearing to read 'Claudia Giuliani', with a large loop at the end.