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

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Abstract

Teucrium chamaedrys 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 to 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.

Three trichome morphotypes were observed, each with a different distribution pattern on the vegetative and reproductive organs: peltate and short-stalked capitate, ubiquitous on the whole plant, and long-stalked capitates, exclusive of the floral whorls. The former and the latter were recognized as the main sites responsible for 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 the sesquiterpenes hydrocarbons and shared the main compounds, *i.e.,* β-caryophyllene, γ-muurolene and β-cubebene. From the literature data, a prevailing defensive action was highlighted at the level of both the vegetative and reproductive organs, along with the potential seductive role played by the floral *bouquet*.

Keywords

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

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 branched rhizome and numerous simple stems, 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", with reference 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 grown ornamentally 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, in case of wound healing and haemorrhoids, to stimulate diuresis and, in more recent time, 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).

As regard to the taxonomy, this species belongs to *Teucrium* sect. *Chamaedrys* (Mill.) Schreb. and included several subspecies distinguished on the base of the leaf features, the general pubescence, and the ecological preferences (Pignatti et al., 2017-2019).

Concerning the micromorphology, the literature proposed 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 regards to the histochemical information on *T. chamaedrys*, a single work reported preliminary and general evidence regarding the chemical nature of the secretory products of the 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 from leaves of Turkish specimens by means of 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 the aerial parts of *T. chamaedrys* of different proveniences 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 to their biological activity, including antimicrobial (Djabou et al., 2013b) and antiphytoviral properties (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 of teucrin A (Bosisio et al., 2004; Nencini et al., 2014).

In this context, the present work 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 sketch a link between the volatile profiles and the potential plant-environment interactions through the literature evaluation of the main compounds' ecological information. Finally, the present 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.

2. Materials and Methods

2.1 Plant material

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 with the codes GBG2020/028 and GBG2020/029, were stored in the *Herbarium* of the Ghirardi Botanic Garden.

2.2 Micromorphology

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

2.2.1 Light Microscopy (LM) and Fluorescence Microscopy (FM)

The epidermal surfaces of the examined organs were preliminarily observed by LM using hand-cut sections. Also, FAA-fixed samples, subsequently dehydrated with ethanol and embedded in Technovit/Historesin were analysed; they were sectioned with a microtome.
The following histochemical dyes were used: Toluidine Blue as a general dye (Beccari and Mazzi, 1966), Fluoral Yellow-088 for total lipids (Brundrett et al., 1991), Nile Red for neutral lipids (Greenspan et al., 1985), Nadi reagent for terpenes (David et al., 1964), PAS-reaction for total polysaccharides (Beccari and Mazzi, 1966), Ruthenium Red for acid polysaccharides (Jensen, 1962), Alcian Blue for mucopolysaccharides (Beccari and Mazzi, 1966), and Ferric Trichloride for polyphenols (Gahan, 1984). Control stainings were simultaneously carried out. Observations were performed under a Leitz DM-RB Fluo optical microscope equipped with a Nikon digital camera.

2.2.2 Scanning Electron Microscopy (SEM)

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

2.3 Phytochemistry

2.3.1 Volatile Organic Compounds (VOCs)

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

HS-SPME Sample analysis – The headspace sampling conditions were as reported in Ascrizzi et al., 2017 (Ascrizzi et al., 2017). For the headspace samplings, Supelco SPME (Solid Phase Micro-Extraction) devices, coated with polydimethylsiloxane (PDMS, 100 μm) were used; the same fibre, preconditioned according to the manufacturer instructions, was employed for all analyses. ~~To ensure a stable temperature, samplings were conducted in an air-conditioned room at 22 ± 1°C; this temperature was chosen to avoid the thermal damage of the plant material and, thus, any artificial-induced volatiles release.~~ After 30 min of equilibration, the fibre was exposed to sample the headspace for further 30 min. Both the equilibration and sampling times were experimentally determined to obtain an optimal adsorption of the volatiles, and to avoid both under- and over-saturation of the fibre and of the mass spectrometer ion trap. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC-MS system. Both the sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each

first SPME extraction and randomly repeated during each series. Quantitative comparisons of relative peak areas were performed between the same compounds in the different samples.

GC-MS analysis - Gas chromatography–electron impact mass spectrometry (GC–EI-MS) analyses were performed with a Varian CP-3800 gas chromatograph (Varian Inc., Walnut Creek, CA, USA) equipped with an Agilent DB-5 (Agilent Technologies Inc., Santa Clara, CA, USA) capillary column (30 m × 0.25 mm; film thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector (Varian Inc., Walnut Creek, CA, USA). Analytical conditions were as follows: injector and transfer line temperatures, 220 and 240 °C, respectively; oven temperature programmed to rise from 60 to 240 °C, at 3 °C min^{-1}; carrier gas, helium at 1 ml min^{-1}; splitless injection. The identification of constituents was based on a comparison of their retention times with those of authentic samples (when available), comparing their linear retention indices relative to a series of pure *n*-hydrocarbons (C5-C25). Computer matching was also used against commercial (NIST 14 and ADAMS) and laboratory-developed library mass spectra built up from pure substances and components of commercial essential oils of known composition and MS literature data (NIST, 2014).

3. Results

3.1 Micromorphological survey

3.1.1 Trichome morphotypes

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

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

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

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

3.1.2 Trichome distribution

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

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

3.1.3 Glandular trichome histochemistry

The results of the histochemical survey are shown in **Table 2** and **Figure 3**. Peltate trichomes produced chemically heterogeneous substances with polysaccharidic, flavonoidic and lipidic components (**Figure 3 a-d**), whereas a hydrophilic secretion (mucopolysaccharides) and an exclusive terpenic secretion were demonstrated for the short- and the long capitates, respectively (**Figure 3 e, f**).

3.2 Phytochemical survey

3.2.1 VOC profile

The HS-SPME analysis revealed on the whole 59 different compounds. In detail, 39 and 45 compounds were identified in the leaf and flower profiles, respectively (**Table 3**).
Sesquiterpene hydrocarbons dominated the leaf profile (93.53%), followed by monoterpene hydrocarbons (2.56%). Oxygenated sesquiterpenes and non-terpene derivatives were present in comparable amounts (1.46%, 1.24%), while oxygenated monoterpenes accounted for 0.85%. β-Caryophyllene (*34*, 27.08%) was the main compound, followed by γ-muurolene (*42*, 22.81%) and β-cubebene (*31*, 12.28%). (*E*)-β-Farnesene (*40*, 6.60%), α-cubebene (*28*, 5.45%), β-bourbonene (*30*, 5.20%) and α-humulene (*39*, 3.75%) showed significant relative abundances, while (*Z*)-γ-bisabolene (*51*), bicyclogermacrene (*44*), β-copaene (*35*), β-pinene (*2*), α-gurjunene (*33*), δ-cadinene (*52*) and dendrolasin (*55*, 1.04%) were detected with percentages between 2.0%-1.0%. The remaining compounds had abundances <1.0% or were present in traces. Fourteen exclusive compounds were identified, all accounting for amounts lower than 1.0% or occurring in traces; the most abundant was α-*neo*-clovene (*38*, 0.51%).
The flower profile was dominated by sesquiterpene hydrocarbons (83.20%), followed by oxygenated monoterpenes (9.10%) and non-terpene derivatives (4.53%). Monoterpene hydrocarbons (2.87%) and oxygenated sesquiterpenes (0.14%) were the least represented classes. β-Caryophyllene (*34*, 38.54) was the most abundant compound, followed by γ-muurolene (*42*, 12.01%) and β-cubebene (*31*, 11.23%). 1,8-Cineole (*6*, 6.67%), α-humulene (*39*, 6.05%), α-cubebene (*28*, 4.26%), α-copaene (29, 3.23%) and β-pinene (*2*, 2.42%)

showed remarkable abundances, while phenylethyl alcohol (*16*), 3-octanone (*3*), (*E*)-β-farnesene (*40*), β-bourbonene (*30*) and methyl carvacrol (*23*) were present between 2.0%-1.0%. The remaining compounds showed percentages <1.0% or were registered in traces. Twenty exclusive compounds were detected, among which α-copaene (*29*, 3.23%) dominated, followed by phenylethyl alcohol (*16*, 1.58%), 3-octanone (*3*, 1.31%) and methyl carvacrol (*23*, 1.07%); the other exclusive compounds were identified with abundances <1.0% or in traces.
The profiles shared 25 common compounds, comprising the 3 dominant ones, *i.e.,* β-caryophyllene (*34*), γ-muurolene (*42*) and β-cubebene (*31*). Moreover, 8 compounds were more abundant in the leaves (*28*, *30*, *33*, *35*, *40*, *44*, *51*, *52*), 3 in the flowers (*2*, *6*, *39*), while the others showed comparable amounts lower than 1.0%.

4. Discussion

The micromorphological investigations on *T. chamaedrys* showed the presence of glandular trichomes, peltate and capitate, typical of the Lamiaceae family (Giuliani and Maleci Bini, 2008). The structure of both morphotypes was consistent with the results of previous works carried out on the vegetative and reproductive organs. The peltate trichomes showed a 4-celled secreting head (Bini Maleci and Servettaz, 1991; Grubešić et al., 2007; Kaya et al., 2009); the short- and long-stalked capitates possessed a bicellular and monocellular head, respectively, matching the morphotypes known in literature: capitates of types I and II in Kaya et al. (2009), different subforms of types B capitates in Grubešić et al. (2007), capitates of type B and C in Bini Maleci and Servettaz (1991), subsessile and clavate tricomes in Ecevit-Genc et al. (2017).
Also the simple, uniseriate and aciculate non-glandular trichomes observed herein were detected in previous studies (Bini Maleci and Servettaz, 1991; Grubešić et al., 2007; Kaya et al., 2009). The observation of the sporadic presence of cuticular ornamentations in the form of micropapillae was also confirmed. Nevertheless, flagelliform coating trichomes were detected on stems by Grubešić et al. (2007) and flask-shaped trichomes were observed on the margins of the calyx teeth by Bini Maleci and Servettaz (1991); both morphotypes were not detected herein. These differences could be related to the high level of infraspecific variability recognized within the target-species.
The distribution pattern of the different trichomes morphotypes proved consistent with literature information, in particular with reference to the exclusive occurrence of the long-stalked capitates on the reproductive organs, with special reference to the calyx (Bini Maleci and Servettaz, 1991; Grubešić et al., 2007; Kaya et al., 2009). The short-stalked capitates were an exception since they resulted sporadic on the corollas, whereas previous contributions indicated them as lacking on this floral whorl.

Concerning the histochemical survey, this work represents the first contribution in which the chemical nature of the secretory products was documented through the use of digital light microscopy techniques. Bini Maleci et al. (1987) reported only generic references 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 capitates, respectively. This evidence matched with 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 resulted exclusively terpene producers (Giuliani et al., 2021, 2020d, 2020a, 2020b). Concerning the short-stalked capitates, the muco-polysaccaridic secretion was widely documented in most of the studied members of the Lamiaceae family (Giuliani and Maleci Bini, 2008). The long-stalked capitates 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 capitates represent the main sites for the terpene secretion at floral level; in addition, also the peltates, widespread on all the epidermal surfaces, 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 to the ease with which 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: β-caryophyllene (*34*), γ-muurolene (*42*) and β-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 α-copaene (*29*) occurred with an appreciable amount (3.23%).

Comparison with previous studies focusing on the VOCs of the target-species was difficult due to the different analytical method employed (Karimi et al., 2011; Özel et al., 2006). However, the sesquiterpenes hydrocarbons were confirmed as the dominant chemical class and β-caryophyllene as the major constituent; the other main molecules were, instead, the alpha isomeric forms of muurolene and cubebene (Karimi et al., 2011; Özel et al., 2006), recorded in our samples with relative percentages equal to 0.37% (*46*, leaves) and 5,45/4.26% (*28*, leaves, flowers), respectively.

The other major compounds did not match those of the Turkish species, for which β-pinene, germacrene D, α-pinene, α-farnesene, α-gurjunene, γ-elemene and γ-cadinene were recorded (Özel et al., 2006). Indeed, in the profiles analyzed herein, results evidenced that germacrene D, α-farnesene and γ-elemene were absent, whereas the remaining compounds showed percentages <3.0% (*1*, *2*, *33*, *50*).

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

Other phytochemical works refer to the analysis of the essential composition of samples of different origin (Bezić et al., 2011; Djabou et al., 2013b; Hajdari et al., 2020; Kaya et al., 2009; Sajjadi and Shookohinia, 2010). Despite the differences related to the analysed plant parts the geographical provenience and the analytical technique, sesquiterpene hydrocarbons invariably prevailed in all the profiles. Nevertheless, β-caryophyllene (*34*) was confirmed as dominant for the Croatian, Iranian and Turkish samples (Bezić et al., 2011; Kaya et al., 2009; Sajjadi and Shookohinia, 2010), whereas in the species from Macedonia germacrene D prevailed (Hajdari et al., 2020). Moreover, the latter was the second most abundant compound in all the profiles from literature. On the contrary, γ-muurolene (*42*) and β-cubebene (*31*) were not detected in any of the other profiles, except for β-cubebene in the Turkish subspecies (subsp. *trapezunticum* 3.2%; subsp. *syspirense* 0.7%) (Kaya et al., 2009).

Regarding the ecological role of the volatilome, the prevalence of the sesquiterpene hydrocarbons in both the vegetative and floral *bouquets* suggested prevailing defensive mechanisms, as widely recognized to this chemical family (Giuliani et al., 2020d). With regards to the dominant compounds, β-caryophyllene (*34*) would develop a repellent action towards adult individuals of pests such as *Tribolium castaneum*, *Lasioderma serricorne* and *Liposcelis bostrychophila* (Cao et al., 2018), as well as parasites and herbivores (Giuliani et al., 2020c). Specifically, as an example, the molecule would be active in plant-herbivore tritrophic interactions, recalling nematodes able to kill parasites such as *Diabrotica virgifera* (Degenhardt

et al., 2009). In addition, β-caryophyllene (*34*) and β-cubebene (*31*) were detected among the main compounds of essential oils with moderate insecticidal activity against *L. serricorne* and *T. castaneum* (Wang et al., 2015). Antifeedant and nematicidal properties were documented for essential oils added with β-cubebene (*31*) (Cao et al., 2018; Sosa et al., 2012). This defensive action would be enhanced by γ-muurolene (*42*) (Chizzola, 2013; Giuliani et al., 2020d) and 1,8-cineole (*6*). Indeed, acaricidal, fumigant and larvicidal properties were recognized to the last compound (Hu et al., 2015; Lucia et al., 2012), as well as antifungal and anti-ochratoxigenic activity against *Aspergillus carbonarius* (Dammak et al., 2019). In addition, 1,8-cineole (*6*), in synergy with β-pinene (*2*), would play a crucial role in allelopathy (Nishida et al., 2005). A defensive action could also be attributed to the most abundant exclusive floral compound, α-copaene (*29*), considering its potential larvicidal property against *Culex quinquefasciatus* (Senthilkumar et al., 2008). Nevertheless, literature information on this compound also revealed a seductive capacity towards ambrosia beetles, vectors of pathogen fungi. Although this action seems to conflict with a defensive role, the emission of such compound could be a "signal" to detect the presence of the pest and, in this way, to prevent the disease onset (Kendra et al., 2016; Owens et al., 2019). Moreover, other defensive mechanisms could be expressed through the emission of (*E*)-β-farnesene (*40*), possessing larvicidal effects against mosquitos of the genera *Anopheles*, *Aedes* and *Culex* (Govindarajan and Benelli, 2016), along with a control role towards aphids (Yu et al., 2012).

However, also the involvement in seductive strategies was recognized to the main compounds. β-Caryophyllene (*34*) would be involved in the preselection and attraction of pollinators (Giuliani et al., 2020c; Novaković et al., 2019). Specifically, its emission from flowers could stimulate positive feedback in honeybees, such as *Apis cerana* (Abraham et al., 2018) and *A. mellifera* (Giuliani et al., 2020e), as well as in non-pollinators such as *Vespa velutina* and *Bombus* spp. (Giuliani et al., 2021; Zhang, 2018). In addition, the high amount of γ-muurolene (*42*) during the flowering stage could be related to the pollination period and to a simultaneous protective action against microorganisms, diseases and herbivores (Fernandez et al., 2021). However, attractive roles towards pollinators were also known for α-humulene (*39*), β-pinene (*2*) and 1,8-cineole (*6*), often in association with β-caryophyllene (*34*) (Abraham et al., 2018; Giuliani et al., 2020b, 2020a; Stevenson, 2019; Zhang, 2018). Finally, β-bourbonene (*30*) was documented as a chemical cue that can drive the foraging preferences of bees (Cellini et al., 2019), while (*E*)-β-farnesene (*40*), abundant in the floral *bouquet* of *Orchis pauciflora*, would contribute to the increase of the foraging rate of pollen grains by *Bombus terrestris* (Valterová et al., 2007).

In this framework, the sharing of the main compounds by the two profiles did not allow to clearly differentiate the potential ecological roles between the vegetative and the floral *bouquets*. However, some hypotheses emerged by evaluating the relative abundance of each molecule. The dual role of β-caryophyllene (*34*) and γ-muurolene (*42*) would suggest an attractive and defensive actions at flower and leaf level, respectively, this latter emphasized by

β-cubebene (*31*). Similar considerations emerged for 1,8-cineole (*6*) and β-pinene (*2*), whose higher relative amounts at floral level may be related to a prevalent attractive role; on the contrary (*E*)-β-farnesene (*40*), more abundant in the leaves, could be related to a predominant defensive action. The attractive role of α-humulene (*39*) and β-bourbonene (*30*) would be equally distributed, since the former is more abundant in the flowers, the latter in the leaves. Finally, the defensive property of α-copaene (29), exclusive of the flowers but not significantly abundant, could be associated with a "warning signal" to prevent the attach by potential parasites.

5. Conclusions

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

Three trichome morphotypes were detected, each with a peculiar distribution pattern on the vegetative and reproductive components. We documented, as an element of novelty, the histochemical nature of the secretory products by means of digital light microscopy techniques. The peltate and long-stalked capitate morphotypes were the main site responsible for the secretion and release of terpenes. In addition, for the first time, the VOC emission profile of plants cultivated in Italy was characterized through HS-SPME, with β-caryophyllene, γ-muurolene and β-cubebene as dominant compounds.

The correlation of the phytochemical results with the ecological role ascribed by the literature to the major compounds allowed us to hypothesize a prevailing defensive role for the vegetative and floral *bouquets*, with a co-occurring potential attractive action at flower level.

Finally, this work enriched the complex of knowledge in the micromorphological, phytochemical and bio-ecological field of the plant heritage preserved at the Ghirardi Botanic Garden, as foreseen within the project "Botanic Garden, factory of molecules".

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Conflicts of interest

The authors declare no conflict of interests.

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Captions to Figures

Figure 1. Trichome morphotypes in *Teucrium chamaedrys* L. **(a, b)** peltate; **(c, d)** short-stalked capitate trichome; **(e, f)** long-stalked capitate trichome. **(a, c, e)** SEM micrographs; **(b, d, f)** LM micrographs, d-e, PAS reaction, f, Toluidine Blue. *Scale bars: a, d = 25 μm; b, c, e, f = 20 μm.*

Figure 2. SEM micrographs showing trichome distribution pattern on the examined vegetative and reproductive organs of *Teucrium chamaedrys* L. **(a, b)** Leaf adaxial (a) and abaxial (b) surfaces with peltates, short capitates and simple non-glandular trichomes. **(c)** Bract abaxial surface with peltates, short capitates and simple non-glandular trichomes. **(d)** Calyx teeth, abaxial surface, with short capitates and simple non-glandular trichomes. **(e, f)** Corolla abaxial surface at the apical region with abundant long capitates. *Scale bars: a-f = 50 μm.*

Figure 3. LM micrographs showing the histochemistry of the glandular trichomes in *Teucrium chamaedrys* L. **(a-d)** peltate: Alcian Blue (a), Aluminiun trichoride (b), Nadi reagent (c), Fluoral yellow-088 (d). **(e)** Short-stalked trichome: Alcian Blue. **(f)** Long-stalked trichome: Nadi reagent. *Scale bars: a-c = 25 μm; d = 50 μm; e-f = 10 μm.*

Figure 1

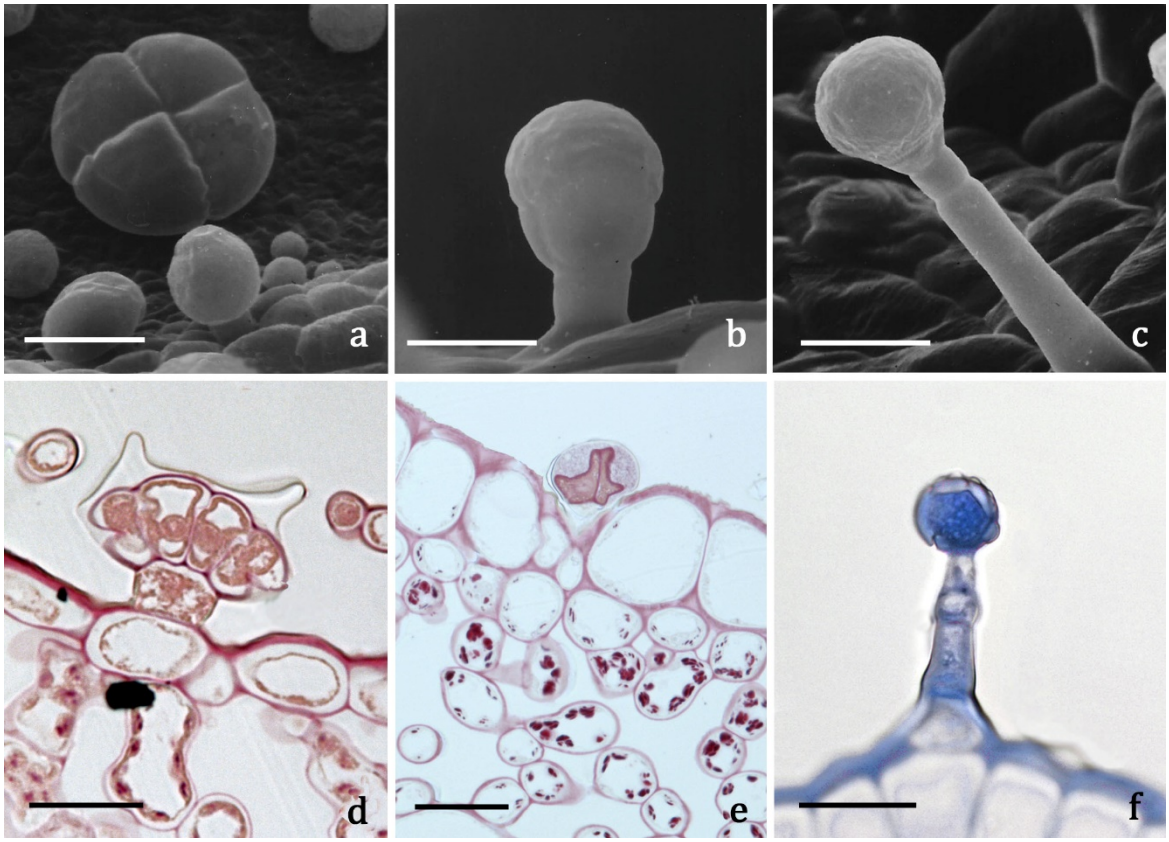

Figure 2

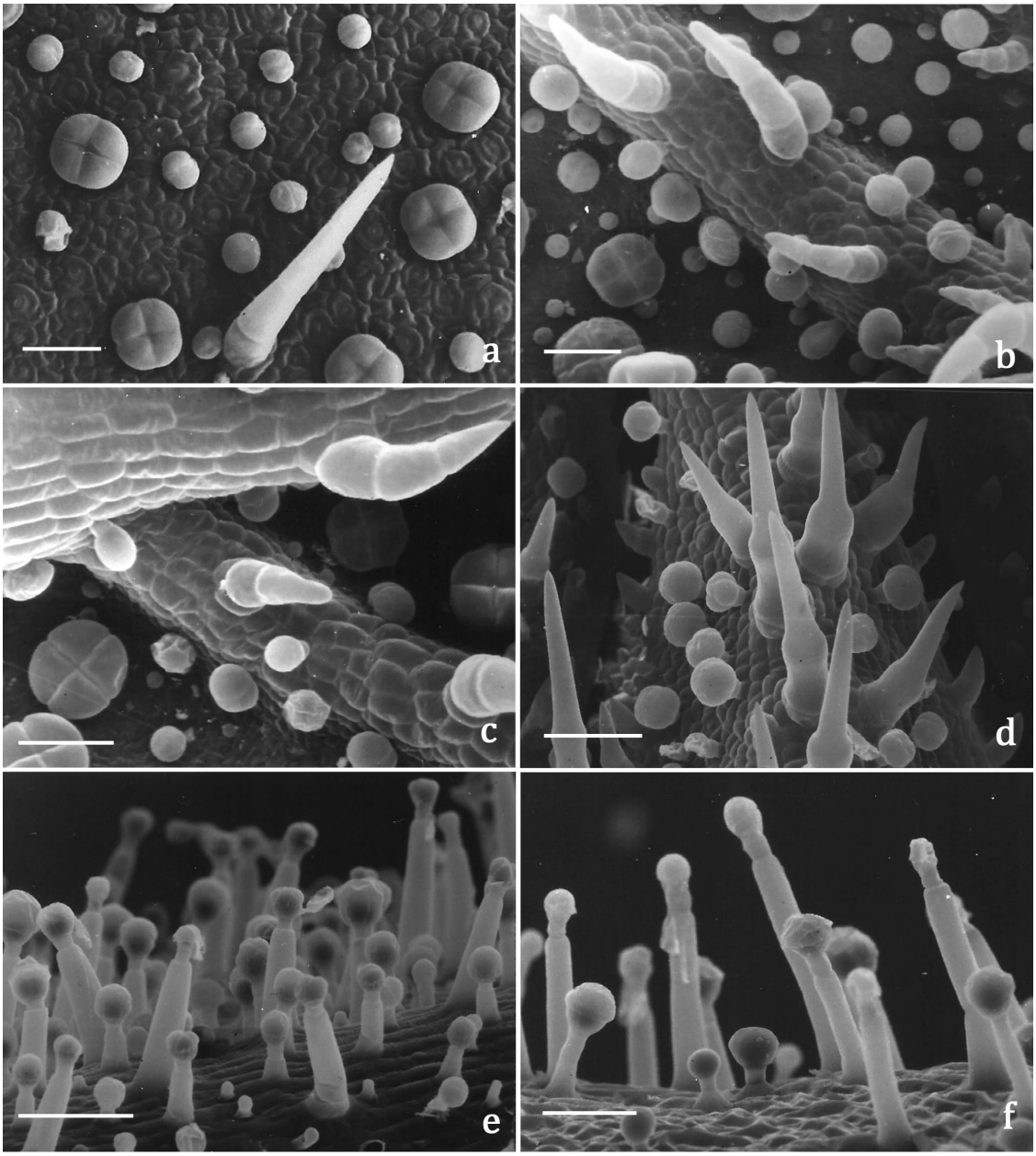

Figure 3

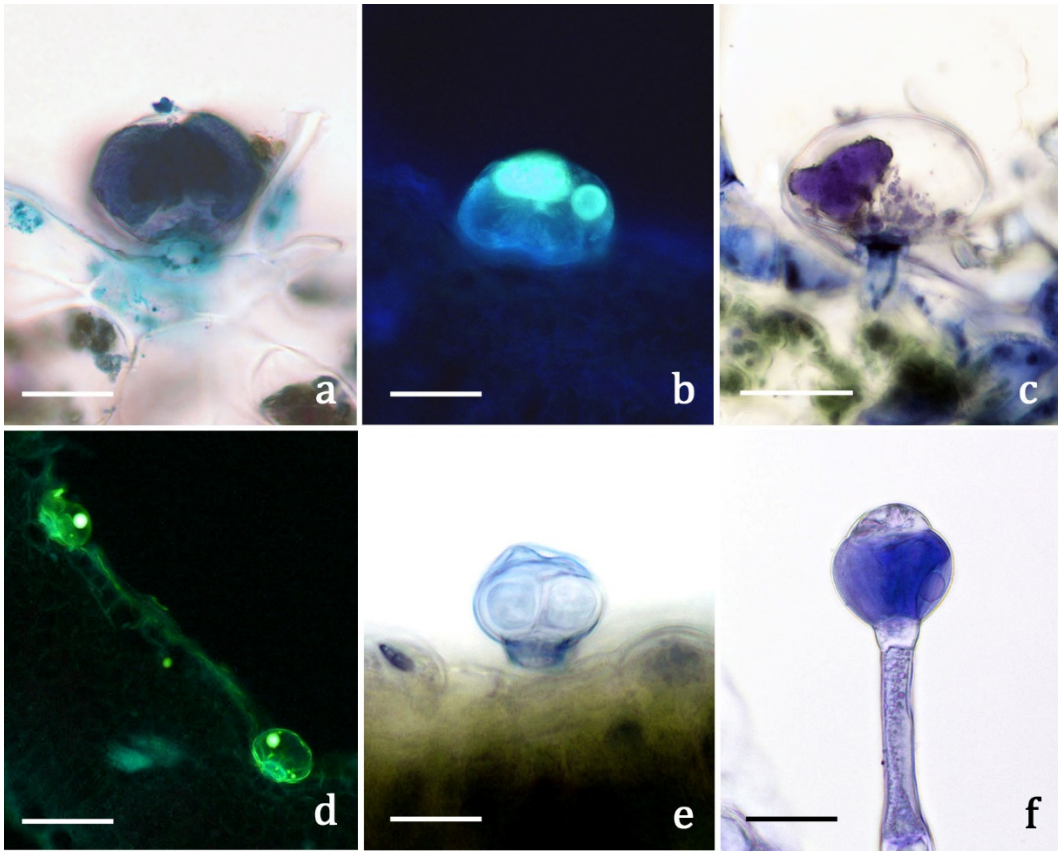

Table 1. Distribution pattern of the glandular and non-glandular trichomes in *Teucrium chamaedrys* L.

Trichome type	Stem	Leaf		Bract		Calyx		Corolla	
		adax	abax	adax	abax	adax	abax	adax	abax
peltate	±	+	++	+	+	-	+	–	++
short capitate	+	+	+	±	+	–	+	–	±
long capitate	–	–	–	–	–	–	+	–	++
simple non-glandular	+	+	+	+	±	+	+	+	+

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

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

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

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

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

	l.r.i.	Compounds	Relative Abundance (%)	
			Leaves	Flowers
1	941	α-pinene	0.92	0.45
2	982	β-pinene	1.40	2.42
3	987	3-octanone	-	1.31
4	1009	(*Z*)-3-hexenol acetate	0.25	-
5	1032	limonene	0.24	tr
6	1034	1,8-cineole (=eucalyptol)	0.73	6.67
7	1052	(*E*)-β-ocimene	tr	-
8	1060	isopentyl butyrate	-	0.12
9	1070	*cis*-sabinene hydrate	-	tr
10	1071	1-octanol	-	0.27
11	1076	*trans*-linalool oxide (furanoid)	-	tr
12	1099	isopentyl-2-methyl butanoate	-	0.24
13	1101	linalool	-	0.24
14	1102	nonanal	tr	-
15	1105	isopentyl isovalerate	-	0.21
16	1110	phenylethyl alcohol	-	1.58
17	1140	nopinone	-	tr
18	1143	camphor	0.12	0.77
19	1178	4-terpineol	-	0.13
20	1199	*n*-dodecane	-	tr
21	1204	decanal	0.14	0.15
22	1240	(*Z*)-3-hexenyl isovalerate	0.14	-
23	1241	methyl carvacrol	-	1.07
24	1244	carvone	tr	-
25	1259	linalool acetate	-	0.22
26	1299	*n*-tridecane	-	0.50
27	1340	δ-elemene	0.29	0.19
28	1351	α-cubebene	5.45	4.26
29	1376	α-copaene	-	3.23
30	1384	β-bourbonene	5.20	1.09
31	1390	β-cubebene	12.28	11.23
32	1399	*n*-tetradecane	0.33	0.15
33	1410	α-gurjunene	1.13	0.46
34	1420	β-caryophyllene	27.08	38.54
35	1429	β-copaene	1.46	0.71
36	1439	α-guaiene	-	0.37
37	1441	aromadendrene	0.26	0.13
38	1454	α-*neo*-clovene	0.51	-
39	ß	α-humulene	3.75	6.05
40	1460	(*E*)-β-farnesene	6.60	1.19
41	1462	*cis*-muurola-4(14),5-diene	0.69	0.43
42	1477	γ-muurolene	22.81	12.01
43	1491	*trans*-muurola-4(14),5-diene	0.36	-
44	1495	bicyclogermacrene	1.61	0.99
45	1497	epizonarene	-	0.28
46	1498	α-muurolene	0.37	-
47	1499	*trans*-β-guaiene	-	0.41
48	1505	δ-amorphene	0.12	-
49	1507	(*E,E*)-α-farnesene	0.24	0.19
50	1513	*trans*-γ-cadinene	tr	0.29
51	1515	(*Z*)-γ-bisabolene	1.80	0.32

52	1524	δ-cadinene	1.06	0.59
53	1534	cadina-1,4-diene	0.31	0.24
54	1538	α-cadinene	0.15	-
55	1574	dendrolasin	1.04	-
56	1575	germacrene D-4-ol	0.29	-
57	1581	caryophyllene oxide	-	0.14
58	1600	*n*-hexadecane	0.38	-
59	1640	*epi*-α-cadinol (=tau-cadinol)	0.13	-
		Monoterpene hydrocarbons	**2.56**	**2.87**
		Oxygenated monoterpenes	**0.85**	**9.10**
		Sesquiterpene hydrocarbons	**93.53**	**83.20**
		Oxygenated sesquiterpenes	**1.46**	**0.14**
		Non-terpene derivatives	**1.24**	**4.53**
		Total	**99.64%**	**99.84%**