Micromorphological and phytochemical survey of Ballota acetabulosa (L.) Benth

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ABSTRACT

• Within the Open Science project entitled ‘Botanic Garden, factory of molecules’, a multidisciplinary study approach was applied to Ballota acetabulosa (L.) Benth., at the Ghiroardi Botanic Garden (Toscolano Maderno, BS, Italy).

• Micromorphological and histochemical investigations were performed on the secreting structures of the vegetative and reproductive organs under light, fluorescence and electronic microscopy. Concurrently the characterization of the volatiles spontaneously emitted from leaves and flowers were examined.

• Four trichome morphotypes were identified: peltate and short-stalked, medium-stalked and long-stalked capitates trichomes, each with a specific distribution pattern. The histochemical analysis was confirmed using ultrastructural observations, with the peltates and long-stalked capitates as the main sites responsible for terpene production. The head-space characterization revealed that sesquiterpene hydrocarbons dominated both in leaves and flowers, with γ-muurolene, β-caryophyllene and (E)-nerolidol as the most abundant compounds. Moreover, a comparison with literature data concerning the ecological roles of the main compounds suggested their dominant roles in defence, both at the leaf and flower level.

• Hence, we correlated the trichome morphotypes with the production of secondary metabolites in an attempt to link these data to their potential ecological roles. Finally, we made the obtained scientific knowledge available to visitors of the Botanic Garden through the realization of new labelling dedicated to B. acetabulosa that highlights the ‘invisible’, microscopic features of the plant.

INTRODUCTION

The genus Ballota L. (Lamiaceae) includes about 35 perennial herbs and subshrubs, native to the temperate regions of Asia, Africa and Europe, with its largest centre of diversity located in the Mediterranean Basin (Morteza-Semnani & Ghanbarimasir 2019). Traditional medicine ascribes to them antiemetic, antispasmodic, sedative, vermifuge and antitussive properties (Yazgan et al. 2010; Morteza-Semnani & Ghanbarimasir 2019).

Ballota acetabulosa (L.) Benth., known as Greek horehound, is a perennial herb native to Greece and the Aegean region, reaching up to 60–80 cm in height, woody at the base, with greyish tomentose stems covered with simple and stellate hairs; middle and upper cauline leaves, crenate-dentate, with a petiole 5–15-mm long; bracteoles linear to spatulate, membranous; the inflorescences are verticillasters, six to 12 flowered; the calyx is 12–15-mm long and the corolla is 15–18-mm long, purple and white in colour (Tutin et al. 1972).

In traditional Greek and Roman medicines, B. acetabulosa was an ingredient in the Dictamnus, the common name for a complex of medicinal species belonging to the Rutaceae and Lamiaceae families, used in the treatment of gynaecological disorders and affections of different origins (Martínez-Francés et al. 2015).

Regarding the morphological and phytochemical characterization of the species, the available contributions are few. The micromorphological literature contains two dated investigations focused on the leaves (Psaras 1986; Psaras & Rhizopoulou 1995), and one study concerning the anatomy of the aerial vegetative organs, in which references to glandular and nonglandular trichomes are reported (Yazgan et al. 2010).

In terms of phytochemical data, there are no studies focused on the profile of the volatile organic compounds (VOC), while several works on the essential oil composition of congeneric species exist, often linked to evaluations of their biological activity (Morteza-Semnani & Ghanbarimasir 2019; Rosselli et al. 2019). Among the most recent works are investigations on B. bullata, B. nigra subsp. uncinata and subsp. poetida, B. undulata, B. saxatilis and B. hispanica, some of which refer to Italian samples (Riccobono et al. 2016; Rigano et al. 2017, 2020; El Mokni et al. 2020). With regard to B. acetabulosa, the literature offers several studies on the characterization of
extracts of increasing polarity in relation to their potential biological activity; moreover, there are studies in which the composition of polyphenolic, flavonoidic and diterpenic content are analysed (Sahpaz et al. 2002; Couladis et al. 2003; Yilmaz & Çitoğlu 2003; Dumlul & Bulut 2007; Dulger & Kilcik 2011a, 2011b; Dulger & Dulger 2012; Askun et al. 2013; Rosselli et al. 2019).

In this context, the present study aims to increase knowledge on *Ballota acetabulosa* in the micromorphological and phytochemical fields through complementary investigative approaches. The primary goals are: (i) description of the morphology and distribution pattern of non-glandular and glandular trichomes by means of light (LM) and scanning electron microscopy (SEM); (ii) analysis, reported here for the first time, of the main compound classes secreted by glandular trichomes through histochemical assays; (iii) study of ultrastructural features of the secreting cells by means of transmission electron microscopy (TEM), in order to identify cellular compartments involved in the secretory process; (iv) characterization, as a novel element, of the VOC spontaneously emitted by leaves and flowers; and (v) correlation of the VOC profiles with potential ecological roles through analysis of literature data concerning the ecological action of the dominant compounds.

Finally, the present work is part of a wider project entitled ‘Botanic Garden, factory of molecules’, investigating a selection of species conserved at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Italy) through a multi-level study using micromorphological and phytochemical approaches in relation to the ecology and biological activity of the secondary metabolites. The achieved results have converged in the realization of new pictorial interpretations dedicated to *Ballota acetabulosa*, where results of the scientific research are made available to the general public.

**MATERIAL AND METHODS**

**Plant material**

*Ballota acetabulosa* is conserved at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Lombardy, Italy), Department of Pharmaceutical Sciences, University of Milan. Samplings for the micromorphological and phytochemical investigations were performed in June 2019. Voucher specimens were labelled with the code GBG2019/027 and deposited in the Herbarium of the Ghirardi Botanic Garden.

**Trichome investigations**

The micromorphology, localization and histochemistry, together with ultrastructural features of trichomes on both vegetative and reproductive organs were studied by means of LM, fluorescence microscopy (FM), SEM and TEM.

We examined at least ten replicates, collected from the same individual, per each plant part to evaluate variability in the micromorphological features. Referring to trichome localization on the examined plant parts, we qualitatively evaluated their distribution using the following symbols: (−) absent, not observed in any of the replicates; (±) sporadic in no more than four replicates; (+) present in all replicates; (+++) abundant in all replicates, with distribution on the whole organ surface.

**Light microscopy (LM)**

Both fresh and fixed samples were analysed. The fresh material was hand-cut into sections 50–60-μm thick; the fixed samples were soaked in FAA solution (formaldehyde/acetic acid/ethanol 70% = 5:5:90) for 7 days, then dehydrated in an ascending ethanol series up to absolute, embedded in Technovit/Historesin and sectioned with a microtome into sections 10–20-μm thick. The following chemicals were used for histochemical staining: Toluidine blue as a general dye (Beccari & Mazzi 1966), Nadi reagent for terpenes (David & Carde. 1964), Ruthenium red for acid polysaccharides (Jensen 1962), Alcian blue for mucopolysaccharides (Beccari & Mazzi 1966) and ferric trichloride for polyphenols (Gahan 1984). Control procedures were concurrently performed.

**Fluorescence microscopy (FM)**

The following histochemical stains were employed on hand-cut fresh samples: Fluoral yellow-088 for total lipids (Brundrett et al. 1991) and Nile red for neutral lipids (Greenspan et al. 1985). Observations were made with a Leitz DM-RB Fluo optical microscope equipped with a Nikon digital camera.

**Scanning electron microscopy (SEM)**

Small segments of leaves, sepals, petals and floral pedicels were fixed in FAA solution, as described above, for 10 days, dehydrated in an ascending ethanol series up to absolute and critical-point dried. The samples were mounted on aluminium stubs, gold-coated and observed under a Philips XL 20 SEM operating at 10 kV.

**Transmission electron microscopy (TEM)**

Small pieces of plant material were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 6.8, and post-fixed in 2% OsO4 in the same phosphate buffer, dehydrated and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The samples were observed under a Philips EM-300 TEM.

**Phytochemistry**

**Volatile organic compounds (VOCs)**

Three fresh leaves and three fresh flowers per plant were cut and immediately inserted into separate glass vials of suitable volume for analysis of VOC.

**Headspace-SPME sample analysis**

The headspace sampling conditions were as reported in Ascrizzi et al. (2017). For the headspace samples, Supelco SPME (Solid Phase Micro-Extraction) devices, coated with polydimethylsiloxane (PDMS, 100 μm) were used. The same new fibre, preconditioned according to the manufacturer’s instructions, was employed for all analyses. To ensure a stable temperature, sampling was conducted in an air-conditioned room at 22 ± 1 °C; this temperature was chosen to avoid thermal damage to the plant material and, thus, release of any artificially induced volatiles. After 30 min of equilibration, the fibre was exposed to sample the headspace for 30 min. Both the equilibration and sampling times were experimentally determined to obtain optimal adsorption of the volatiles, and
Gas chromatography–electron impact mass spectrometry analysis (GC–EI-MS)

The GC–EI-MS analyses were performed with a Varian CP-3800 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with an Agilent DB-5 (Agilent Technologies, 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). Analytical conditions were as follows: injector and transfer line temperatures, 220 °C and 240 °C, respectively; oven temperature programmed to rise from 60 °C 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 as well as MS literature data (NIST, 2014).

RESULTS

Trichome morphotypes and distribution patterns

Leaves and flowers of B. acetabulosa displayed an indumentum of non-glandular and glandular trichomes, the latter including both peltate and capitate types (Table 1, Fig. 1a–f). The peltate trichomes were composed of a basal cell, a stalk cell and a large secretory head with eight cells arranged in a single disc. They were localized throughout the whole plant epidermis, being scarce on leaves and common on the sepal and petal abaxial surfaces (Table 1, Fig. 1c–e).

Three different types of capitate trichome were found: short-stalked consisting of a basal cell, a stalk cell and a round two- to four-celled head, mainly located along the vein system of the whole plant, especially on the leaf and corolla abaxial sides (Table 1, Fig. 1a–c, e); medium-stalked consisting of a basal cell, one to two stalk cells, a neck cell and a one- to two-celled head, occurring on leaves and the calyx abaxial side (Table 1, Fig. 1a–c); and long-stalked, consisting of a basal cell, two to four stalk cells, a neck cell and a globose two- to four-celled secretory head. These hairs were primarily distributed on the leaf adaxial side, on the sepal abaxial side and on the floral peduncle (Table 1, Fig. 1b–d, f).

The observed non-glandular hairs were of two different types. The first was simple, multicellular, uniseriate with distinct articulation between the cells (Fig. 1a–c, f), distributed on surfaces of both the vegetative and reproductive organs (Table 1). The second was dendritic multicellular hairs with

Table 1. Distribution pattern of the glandular and non-glandular trichomes on abaxial (abax) and adaxial (adax) surfaces of Ballota acetabulosa (L.) Benth.

| Trichome type   | Leaf       | Calyx      | Corolla    | Floral peduncle |
|-----------------|------------|------------|------------|-----------------|
|                 | adax      | abax      | adax      | abax            |
| peltate         | ±          | ±          | –          | ++              |
| short capitate  | +          | +          | +          | + +             |
| medium capitate | +          | – ±        | ±          | – –             |
| long capitate   | ±          | – ±        | +          | – –             |
| simple          | ++ – – ±   | ±          | + – +      | – –             |
| non-glandular   | – – – ±    | +          | +           | +               |
| dendritic       | –          | +          | –           | –               |
| non-glandular   | – – – ±    | +          | – –         | –               |

Symbols: (−) missing, (±) sporadic, (+) present, (+++) abundant.

To avoid both under- and over-saturation of the fibre and of the mass spectrometer (MS) ion trap. Once sampling was complete, 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 samples. Furthermore, control 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.

Phytochemistry

Volatile organic compounds (VOCs)

The HS-SPME analysis on leaves and flowers of B. acetabulosa revealed 46 compounds. In detail, 34 and 21 compounds were identified on surfaces of both the vegetative and reproductive organs (Table 1).
Almost all of the leaf profile was dominated by sesquiterpene hydrocarbons (96.61%), followed by oxygenated sesquiterpenes (2.37%) and non-terpene derivatives (0.12%). The most abundant compound was $\gamma$-muurolene (compound 31, 43.63%), followed by $\beta$-caryophyllene (19, 21.97%). ($E$)-$\beta$-Farnesene (28, 5.12%), $\beta$-bourbonene (13, 3.54%), valencene (33, 3.19%), alloaromadendrene (29, 2.53%), $\alpha$-copaene (11, 2.22%), $\beta$-copaene (20, 2.16%) and carotol (44, 2.04%) showed percentage relative abundances in the range 5.0–2.0%, while $\beta$-elemene (15, 1.96%), geijerene (3, 1.60%), ($E,E$)-$\alpha$-farnesene (36, 1.35%) and cis-muurola-4(14),5-diene (30, 1.02%) in the range of 2.0–1.0%. The remaining compounds occurred at less than 1.0% or in trace amounts (<0.1%).

A total of 25 exclusive compounds were identified, among which valencene (33, 3.19%), alloaromadendrene (29, 2.53%), $\beta$-copaene (20, 2.16%) and carotol (44, 2.04%) were the most abundant. The remaining exclusive compounds accounted for less than 2.0% or trace amounts (<0.1%).

Sesquiterpene hydrocarbons represented the main compound class in the floral profile, (45.86%), followed by...
non-terpene derivatives (21.85%), oxygenated sesquiterpenes (17.14%) and apocarotenoids (11.21%), while the monoterpene class contained only oxygenated compounds (0.99%).

\(\gamma\) Muurolene (31, 25.33%) was the dominant compound, followed by (\(E\))-nerolidol (41, 17.14%), \(\beta\)-caryophyllene (19, 6.76%), (\(E\))-geranyl acetone (26, 6.65%) and 1-nonanal (2, 5.28%). \(\gamma\)-Elemene (21, 3.78%), (\(E\))-\(\beta\)-ionone (32, 3.27%), aromadendrene (25, 3.12%), \(\alpha\)-copaene (11, 2.65%), \(\beta\)-elemene (19, 2.63%), n-heptadecane (46, 2.34%), n-tetradecane (16, 2.04%) and n-hexadecane (45, 2.01%) showed abundances in the range 3.0–2.0%, while n-pentadecane (35, 1.79%), \(\beta\)-cubebene (14, 1.59%) and cis-\(\alpha\)-ambrinol (24, 1.29%) were detected in relative abundances ranging between 2.0% and 1.0%. The other compounds were present at percentages below 1.0% or in trace amounts (<0.1%). Twelve exclusive compounds were detected, among which (\(E\))-nerolidol (41, 17.14%) and (\(E\))-geranyl acetone (26, 6.65%) were the most abundant. The remaining compounds were detected at percentages in the ranges 3.0–2.0%, 2.0–1.0% and <1.0%.

Leaves and flowers shared nine common compounds: \(\gamma\)-muurolene was most prevalent (31, 43.63% leaves; 25.33% flowers), followed by \(\beta\)-caryophyllene (19, 21.97% leaves; 6.76% flowers), both with a higher percentage in leaves than in flowers. (\(E\))-\(\beta\)-Farnesene (28) and \(\beta\)-bourbonone (13) were more abundant in the leaves (5.12%; 3.54%), while in flowers they were only detected in trace amounts (<0.1%). In contrast, n-decanal (5) and n-nonanal (2) revealed higher percentages in flowers (7.51%; 5.28%) than in leaves (0.12%; traces, <0.1%), while the relative abundances of compounds (11), (14) and (15) were between 2.0–1.0% and they were quantitatively comparable in the two profiles.

Scientific dissemination

The scientific results reported in the “Trichomes” and “Phytochemistry” sections converged in the realization of a new pictorial explanatory approach for *B. acetabulosa* at the Ghirardi Botanic Garden (Toscolano Maderno, BS, Italy). In addition to
Table 3. HS-SPME profiles of the leaves and flowers of *Ballota acetabulosa* (L.) Benth.

| L.r.i. | Compounds                          | Relative Abundance (%) | Leaves | Flowers | Standard availability | Chemical class |
|-------|------------------------------------|------------------------|--------|---------|-----------------------|----------------|
| 1     | 1,8-cineole                        | tr                     | –      | A       | OM                    | OM            |
| 2     | 1-nonanal                          | tr                     | 5.28   | A       | FA                    | FA            |
| 3     | geijerene                          | 1.6                    | –      | NA      | SH                    | SH            |
| 4     | n-dodecane                         | tr                     | –      | A       | FA                    | FA            |
| 5     | 1-decanal                          | 0.12                   | 7.51   | A       | FA                    | FA            |
| 6     | methyl carvacrol                   | –                      | 0.99   | A       | OM                    | OM            |
| 7     | 1-tridecene                        | tr                     | –      | A       | FA                    | FA            |
| 8     | geijerene                          | 0.19                   | –      | NA      | SH                    | SH            |
| 9     | n-dodecane                         | 0.32                   | –      | NA      | SH                    | SH            |
| 10    | cyclosativene                      | tr                     | –      | NA      | SH                    | SH            |
| 11    | 1,8-copaene                        | 2.22                   | 2.65   | A       | SH                    | SH            |
| 12    | daucene                            | 0.69                   | –      | NA      | SH                    | SH            |
| 13    | β-bourbonene                       | 3.54                   | tr     | NA      | SH                    | SH            |
| 14    | β-cubebene                         | 0.96                   | 1.59   | NA      | SH                    | SH            |
| 15    | β-elemene                          | 1.96                   | 2.63   | A       | SH                    | SH            |
| 16    | n-tetradecane                      | –                      | 2.04   | A       | FA                    | FA            |
| 17    | α-gurjunene                        | 0.26                   | –      | A       | SH                    | SH            |
| 18    | colnames="col3">1,7-di-epi-β-cedrene | 0.18                 | –      | NA      | SH                    | SH            |
| 19    | β-caryophyllene                    | 21.97                  | 6.76   | A       | SH                    | SH            |
| 20    | β-copaene                          | 2.16                   | –      | M       | SH                    | SH            |
| 21    | γ-elemene                          | –                      | 3.78   | NA      | SH                    | SH            |
| 22    | trans-β-bergamotene                | 0.85                   | –      | M       | SH                    | SH            |
| 23    | α-guaiene                          | 0.82                   | –      | NA      | SH                    | SH            |
| 24    | cis-α-ambrinol                     | –                      | 1.29   | NA      | AC                    | AC            |
| 25    | aromadendrene                      | –                      | 3.12   | A       | SH                    | SH            |
| 26    | (E)-geranyl acetone                | –                      | 6.65   | A       | AC                    | AC            |
| 27    | α-neo-clovene                      | 0.12                   | –      | NA      | SH                    | SH            |
| 28    | (E)-β-farnesene                    | 5.12                   | tr     | A       | SH                    | SH            |
| 29    | alloaromadendrene                  | 2.53                   | –      | M       | SH                    | SH            |
| 30    | cis-muurola-4(14),5-diene           | 1.02                   | –      | NA      | SH                    | SH            |
| 31    | γ-muurolene                        | 43.63                  | 25.33  | M       | SH                    | SH            |
| 32    | (E)-β-ionone                       | –                      | 3.27   | A       | AC                    | AC            |
| 33    | valencene                          | 3.19                   | –      | A       | SH                    | SH            |
| 34    | α-muurolene                        | 0.36                   | –      | NA      | SH                    | SH            |
| 35    | n-pentadecane                      | –                      | 1.79   | A       | FA                    | FA            |
| 36    | (E,E)-α-farnesene                  | 1.35                   | –      | M       | SH                    | SH            |
| 37    | trans-γ-cadinene                   | 0.47                   | –      | NA      | SH                    | SH            |
| 38    | δ-cadinene                         | 0.98                   | –      | NA      | SH                    | SH            |
| 39    | lilial                             | –                      | 0.88   | A       | FA                    | FA            |
| 40    | α-cadinene                         | 0.12                   | –      | NA      | SH                    | SH            |
| 41    | (E)-nerolidol                      | –                      | 17.14  | A       | OS                    | OS            |
| 42    | germacrene D-4-ol                  | 0.12                   | –      | NA      | OS                    | OS            |
| 43    | caryophyllene oxide                | 0.21                   | –      | A       | OS                    | OS            |
| 44    | carotol                            | 2.04                   | –      | M       | OS                    | OS            |
| 45    | n-hexadecane                       | –                      | 2.01   | A       | FA                    | FA            |
| 46    | n-heptadecane                      | –                      | 2.34   | A       | FA                    | FA            |

**Note:**

- **Oxygenated monoterpenes**
- **Sesquiterpene hydrocarbons**
- **Oxygenated sesquiterpenes**
- **Apocarotenoids**
- **Fatty acid derivatives**

**Abbreviations:**
- **OM:** Oxygenated monoterpene
- **SH:** Sesquiterpene hydrocarbon
- **OS:** Oxygenated sesquiterpene
- **AC:** Apocarotenoid
- **FA:** Fatty acid derivative

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*Ballota acetabulosa: trichomes and volatiles* 
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*Oxygenated monoterpenes* 0.99
*Sesquiterpene hydrocarbons* 96.61
*Oxygenated sesquiterpenes* 2.37
*Apocarotenoids* 11.21
*Fatty acid derivatives* 21.85

**Total** 99.10% 97.05%
the plant macroscopic features, this highlights the microscopic morphology, volatile composition of the vegetative and floral bouquets, and noteworthy data on the plant–environment interactions (Fig. 3).

**DISCUSSION**

Based on their morphology, the glandular trichomes of *B. acetabulosa* corresponded to the two main types that occur in the Lamiaceae: peltate and capitate hairs.

The peltate hairs were widespread on the examined vegetative and reproductive organs, being scarce on leaves and abundant on sepals; they exhibited an eight-celled head, as already described in *Scutellaria brevibracteata* subsp. *subvelutina* (Rech. f.) Greuter & Burdet (Giuliani et al. 2020a). The secreted substances were stained intensely only in response to the lipophilic dyes, especially the Nadi reagent, indicating that the secretion was exclusively composed of terpenes. Therefore, these hairs were typical essential oil producers, as found in many Lamiaceae (Werker 2000), although in several species of this family chemical complexity of the secretion has also been reported (Giuliani & Maleci Bini 2008). The ultrastructural study on secretory cells confirmed the histochemical evidence; indeed, the combination of SER and multi-shaped electron-dense plastids with few internal membrane systems is very common for terpenoid-producing cells (Hallahan 2000). The combination of SER with electron-dense plastids can be considered a sign of terpenoid production, even if the role of the SER is not yet completely understood (Levine 2004; Giuliani et al. 2017). The transfer of terpenoids from one organelle to another and, eventually, to the plasma membrane is mediated by membrane contact (Levine 2004). The membrane contacts between electron-dense plastids and SER observed here may thus be considered an indication of the activity of terpenoid production.

*Ballota acetabulosa* presented three types of capitate hairs, which greatly differed in overall size, stalk length and shape of the secretory head. The short capitate trichomes were widespread and are the only type common to all Lamiaceae species examined so far (Giuliani & Maleci Bini 2008). The hydrophilic procedures only gave faint positive reactions, indicating the exclusive presence of muco-polysaccharides (Giuliani & Maleci Bini 2008). In most Lamiaceae species examined so far, these trichomes are rich in polysaccharides; nevertheless, in

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**Fig. 3.** New labelling of *Ballota acetabulosa* (L.) Benth. at the Ghirardi Botanic Garden (University of Milan, Toscolano Maderno, Brescia, Italy).
several members of Stachys subgenus Betonica (L.) Bhattacharjee and in Stachys annua (L.) L. the secretion is more complex in composition, containing both terpene and polyphenolic fractions (Giuliani & Maleci Bini 2008).

The medium capitate trichomes corresponded to the capitule type 2 hairs described in several members of the genera Salvia, Stachys, Sideritis and Scutellaria (Giuliani & Maleci Bini 2008; Giuliani et al. 2020a, 2020b). The resulting secretion was heterogeneous, since it stained positively in both lipophilic and hydrophilic tests. The trichome ultrastructure displayed dense cytoplasm with numerous small vacuoles, electron-dense plastids, few Golgi bodies and RER, confirming the histochemical results.

The long capitule trichomes were scarce on leaves and numerous on the calyx abaxial surface. Noteworthy, these capitule morphotypes were distributed on leaves in our samples, while in the other Lamiaceae species examined so far they were found only on reproductive organs (Giuliani & Maleci Bini 2008). The secreted material stained positively for all the employed staining procedures, revealing the production and release of a heterogeneous secretion consisting of lipid, polysaccharide and phenolic compounds. Secretory cell ultrastructure supports the histochemical observations; indeed, at the beginning of the secretory phase, the head cells showed mitochondria, Golgi bodies and well-developed RER elements, with plastids containing some starch grains. Plastids are also involved in the production of polyphenol precursors, and the key enzymes for their biosynthesis are localized in the starch granules (Grundhöfer et al. 2001). In mature trichomes, Golgi bodies and RER occurred only occasionally, while the most striking ultrastructural features were multi-shaped plastids sheathed by periplastidial SER. These ultrastructural features may indicate the initial production of hydrophilic substances and polyphenols, followed by the release of terpenes at a later stage of the secretory process (Giuliani & Maleci Bini 2008).

The observed glandular trichome morphotypes were consistent with those previously reported for the target species from Turkey (Yazgan et al. 2010), although a comparison of the distribution pattern was not possible, and from several congeneric species from Egypt (Osman 2012). Regarding the non-glandular indumentum, our observations confirmed the occurrence of simple uniseriate trichomes on the whole plant epidermis and of dendritic trichomes exclusively on the reproductive organs. However, we did not detect the stellar morphotype, previously reported in B. acetabulosa (Tutin et al. 1972; Yazgan et al. 2010) and in other congeneric species (Osman 2012). This could be significant and was probably related to the cultivation conditions in the study area, which could have affected the features of the non-glandular indumentum.

Concerning the phytochemical investigations, the foliar profile was more complex than the floral profile, as also evidenced by the presence of a higher number of exclusive compounds, compared to that of the flowers (25 versus 12). Moreover, only nine common compounds were detected, among which the most abundant were: γ-muurolene, with a relative percentage higher in leaves than in flowers (31, 43.63% leaves; 25.33% flowers), and β-caryophyllene, the second most abundant compound in leaves (19, 21.97%), while in flowers it was less abundant (19, 6.76%). As for the exclusive compounds, valencene (33, 3.19%) was the most abundant in leaves, while in flowers (E)-nerolidol (41, 17.14%) was most prevalent.

In general, in the case of Lamiaceae species, flowers emit more compounds than leaves. However, the opposite situation is not uncommon. For example, the leaf profile was more complex than the floral profile in Scutellaria alissina (Giuliani et al. 2020b), Salvia mirzayanii (Majid, 2015) and Lamium hybridum (Flamini et al., 2005).

The comparison between phytochemical and histochemical results confirms the terpene production, in particular on the petal abaxial surface, where petalate trichomes occurred, and on the calyx and floral peduncle, where long-stalked capitules were detected. In contrast, there was no such marked correspondence for the leaves, where terpene productivity appeared to be related to the presence of medium-stalked capitules. Due to the absence of studies on the VOC and essential oil (EO) characteristics of the target species, only a comparison with some congeneric species is possible. Specifically, studies carried out in Italy investigated the EO composition of B. hispanica, B. nigra subsp. uncinita and subsp. foetida, B. undulata and B. saxatilis (Fraternale et al. 2009; Fraternale & Ricci 2014; Riccobono et al. 2016; Rigano et al. 2017, 2020). Despite the detailed technical approaches used in this study, the volatile profile provided suggests the need for further studies on B. acetabulosa volatile profile using in vivo techniques.

Concerning the ecological role(s) of the main compounds detected in the VOC profiles, it emerged that the most abundant compound, γ-muurolene (31), has a role in defence, and is the most common sesquiterpene hydrocarbon (Chizzola 2013; Giuliani et al., 2020b). Although it was detected as a common compound, its high relative abundance in the leaves allowed us to hypothesize a marked repellant action at the vegetative organ level. Regarding another common compound, β-caryophyllene (19), is known to act in both attraction and repulsion (Abraham et al. 2018; Zhang 2018; Lobo et al. 2019; Giuliani et al. 2020a, 2020b). Indeed, it attracts honeybees such as Apis cerana (Abraham et al. 2018), non-pollinators such as Vespa velutina and species in the genus Bombus (Zhang 2018), but it provides defence against parasites such as Diaphania hyalinata (Lobo et al. 2019), hemiptera and lepidoptera in the genera Apolygus, Aphis and Helicoverpa (Zhang et al. 2020). Therefore, in our case study, we hypothesize that this compound has an attraction role in flowers and a repellant role in leaves. The plants must face two simultaneous and conflicting issues: the need to advertise their pollen as a reward for pollinators and the necessity to defend it from predators.

Concerning the leaf exclusive compounds, alloaromadrene (29), together with compounds including β-copaene (20), have larvicidal activity towards Culex quinquefasciatus (Senthilkumar et al. 2008). In addition, an ecological survey conducted at the community level, it was documented that the abundance of this compound increases in cases of interspecific competition (Ormeño et al. 2007).

Referring to the floral bouquet, the overall abundance of sesquiterpene hydrocarbons makes it difficult to exclude a defence action even at the flower level. To this end, the exclusive compound (E)-nerolidol (41) represents a powerful signal that stimulates expression of plant defences. In fact, its production activates a consequential series of cellular and molecular reactions, leading the plant to synthesize and accumulate a significant amount of broad-spectrum defence molecules against herbivores and several types of parasite (Chen et al. 2020). Nerolidol is synthesized in many plants as an intermediate in
the production of a herbivore-induced terpenoid, (E)-4,8-dimethyl-nonatriene (DMNT) (Chan et al. 2016). Moreover, it could even develop an antibacterial action together with β-farnesene (28) (Kiryu et al. 2018), and also act as a nematicide (El-Habashy et al. 2020).

In addition, (E)-geranyl acetone (26) is involved in several VOC-mediated tritrophic interactions (Morawo et al. 2016; Pinto-Zevallos et al. 2018; Giuliani et al. 2020a, 2020b). This suggests the need to accumulate potential defence compounds against target species of flower parasites. Overall, the data stimulated our curiosity for future investigations on possible parasites known from the literature in relation to B. acetabulosa, as information is lacking on such aspects.

Finally, our study, which originated and takes place at the Ghirardi Botanic Garden, converged towards the fruition of research products available to the general public, following a new approach for dissemination of scientific data that is increasingly available in the literature (Giuliani et al. 2020, 2020a, 2020b).

CONCLUSION

Our work on B. acetabulosa was based on a multidisciplinary approach. In the micromorphological survey, the occurrence of pellet trichomes and of three capitate morphotypes emerged, each characterized by a specific distribution pattern on the vegetative and reproductive organs. The application of histochemical tests was an element of novelty for the target species; the results were confirmed by the ultrastructural observations, corroborating that the petals and long-stalked capitules are mainly responsible for terpenes production, finally resulting in the spontaneous emission of volatiles, as characterized through head-space analysis in the phytochemical survey. In the vegetative and floral bouquets, the dominance of sesquiterpene hydrocarbons emerged, among which γ-muurolene, β-caryophyllene and (E)-nerolidol were the dominant compounds. Moreover, a comparison with literature data on the ecological roles of the most abundant compounds allowed us to hypothesize a prevailing defence action of the volatile emissions, both at the leaf and flower level. These data represent a new focus in the research pathway towards characterization of the Ghirardi Botanic Garden as a ‘factory of molecules’, an innovative vision placed in an Open Science context.

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