Growing underwater basil in Nemo's Garden®: phytochemical, physiological and micromorphological analyses

Roberta ASCRIZZI[a,*], Claudia GIULIANI[b], Laura PISTELLI[c,d], Claudio CERVELLI[e], Barbara RUFFONI[e], Elisabetta PRINCI[f], Gianni FONTANESI[f], Guido FLAMINI[a,d] and Luisa PISTELLI[a,d]

[a]Dipartimento di Farmacia, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy; [b]Dipartimento di Scienze Farmaceutiche (DISFARM), Università di Milano, Via Mangiagalli 25, 20133 Milan, Italy; [c]Dipartimento Scienze Agrarie, Alimentari e Agro-Ambientali (DISAA-a), Via del Borghetto 80, 56124 Pisa, Italy; [d]Centro Interdipartimentale di Ricerca "Nutraceutica e alimentazione per la salute" NUTRAFOOD, Via del Borghetto 80, 56124 Pisa, Italy; [e]CREA-Research Centre for Vegetable and Ornamental Crops, Corso Inglesi 508, 18038 Sanremo (IM), Italy; [f]Ocean Reef Group, Via Arvigo 2, 16010 Sant'Olcese (Genova), Italy

*Corresponding Author. E-mail: roberta.ascrizzi@for.unipi.it.

Highlights

1. Nemo's Garden® underwater biospheres are a green, alternative agriculture system
2. Underwater basil did not show micromorphological changes of the leaf *indumentum*
3. Relevant differences were detected in the essential oil and head space compositions
4. More photosynthetic pigments and polyphenols were produced in the underwater plants
5. Basil seems well adapted: studies on other species are needed to evaluate a scale-up

Abstract

The need for alternative cultivation methods is urgent for regions of the world where cultivable areas are scarce: underwater areas are unexploited and vast. Nemo's Garden® Project aims at creating a green, alternative agriculture system: its biospheres are underwater greenhouses, developed for areas where plants growth is difficult in terrestrial conditions.

Basil was chosen as model plant to study its phytochemical, physiological, and micromorphological characteristics in comparison with the same plants grown in terrestrial conditions in a CREA greenhouse.

While the micromorphological analyses show no detectable differences between the control and the biospheres samples, the phytochemical investigations evidenced a switch of the essential oil chemotype. The head-spaces were also different: sesquiterpenes dominated the biospheres samples, whereas oxygenated monoterpenes accounted for half the control sample emission. Differences also emerged in the physiological investigation: chlorophylls, carotenoids and polyphenols were present in higher amounts in the biospheres samples, with an increased antioxidant activity.

Keywords: *Ocimum basilicum*; essential oil; HS-SPME; volatiles; glandular trichomes; photosynthetic pigments; carotenoids; antioxidant activity; subaqueous biospheres

1. Introduction

Ocimum basilicum L. is an annual culinary herb of the Lamiaceae family. Whilst it is native to Asia, it is largely grown in Italy and it is extensively used in the Italian cuisine, in which it represents the main ingredient of the Ligurian Pesto sauce. The trade importance of *O. basilicum* is relevant, as the 'Genovese' variety has been conferred the PDO (Protected Designation of Origin).

Besides the traditional use in food, basil has been widely utilized as a flavoring agent, in perfumery and medical industry (Grayer et al., 2004; Özcan, Arslan, & Ünver, 2005; Politeo, Jukic, & Milos, 2007). The leaves and flowering tops of the plant are perceived as carminative, galactogogue, stomachic and anti-spasmodic in folk medicine and its essential oil showed antimicrobial and antioxidant activities (Hussain, Anwar, Hussain Sherazi, & Przybylski, 2008). Moreover, basil contains phenolic antioxidant compounds, free radical-scavengers, and metal chelators (Sgherri, Cecconami, Pinzino, Navari-Izzo, & Izzo, 2010). Beyond culinary consumption, aromatic herbs such as basil are important sources of value-added products like essential oils (EOs), which are used in many industrial branches (i.e. pharmaceutical, cosmetic, pest management, etc.). As estimated in the UNIDO and FAO 2005 Report (United Nations Industrial Development Organization and Food and Agriculture Organization, 2005), approximately 43 tons of basil EO are traded annually, with a total trade value of 2800000 $. The October 2016 Market Insider Report on Essential Oils and Oleoresins of the International Trade Center (INTRACEN, 2016) presents the prices per kg of basil essential oil based on its geographical origin and production method (see Table 1).

The FAO climate biome classification (http://ecocrop.fao.org) reports basil as a species which tolerates well tropical and subtropical climates, both in wet and humid conditions, and oceanic climate: differences in the growth conditions lead to the development of different chemotypes of basil, each of them with a characteristic aroma and taste determined by a pool of several compounds (Lee, Umano, Shibamoto, & Lee, 2005).

Such an adaptable species, with a large worldwide use and added-value sector interest, is a viable candidate as a crop to invest in, even in developing countries. However, seasonal changes lead to variability in the contents of most of the chemical constituents (Hussain et al., 2008). Also, the light irradiation can contribute

to changes in metabolic compounds: UV-B and blue light affect the generation of phenolic compounds in basil (Shiga et al., 2009).

To overcome the lack of cultivable areas, Ocean Reef Group developed the Nemo's Garden® Project, looking at new branches of green and blue economy. Nemo's Garden® may represents an alternative system of agriculture, particularly useful for herbal crops, especially dedicated to those areas where environmental conditions, economical or geo-morphological reasons make plants growth extremely difficult (Princi et al., 2016). The technology developed in the framework of Nemo's Garden® Project consists of underwater greenhouses called 'biospheres' (Dini, Princi, Gamberini, & Gamberini, 2016). They are air-filled domes made of acrylic (transparent plastic material) that are anchored to the bottom of the sea by many chains, floating from 5 to 10 meters depth in front of the shoreline of the Noli town, close to Savona, Italy. They hold approximately 2000 liters of air. Nemo's Garden® Project started in 2012, but since 2015 a systematic study on the characteristics of plants grown underwater has been started to understand the effect of the marine environment on them. Several plant species were cultivated in the Nemo's Garden® biospheres. Basil was chosen as model plant to study its phytochemical, physiological, and micromorphological characteristics in comparison with plants of the same variety grown in a terrestrial environment in the CREA Centre at Sanremo (Imperia, Liguria, Italy) greenhouses, very close to Noli. The aim of the present study was the evaluation of the micromorphological, phytochemical and physiological responses to this environment, where the terrestrial greenhouse is substituted by an underwater biosphere.

Nemo's Garden® represents a very promising project, an attempt to answer the urgent need for new agriculture systems: further studies are needed to assess the effects that these new environmental conditions exert on different crops.

2. Materials and methods

2.1. Plant material and growth conditions

2.1.1. Control samples

The control samples were sown at CREA in Sanremo on 19 August 2015 in 1-liter plastic pots filled with mineral wool and coconut fiber 50:50 v/v. The plants were grown in a greenhouse until 15 October 2015,

when the samples were collected. Fertigation was accomplished every 1-2 days with a nutrient solution containing $N:P_2O_5:K_2O$=1:0.7:1 and microelements. Inside the greenhouse the maximum daily light intensity ranged between 23000 and 35000 lux (605-920 µmol/m^2/s), the daily temperature between 18.0 and 30.0 °C (with a mean ΔT of 7.0 °C). The mean daily relative humidity ranged between 42 and 63%, depending on the day. Samples were collected and used either fresh or dried at natural room conditions.

2.1.2. Nemo's Garden® samples

In the underwater farm, seeding occurred on 25 August 2015. Seeds were sown in slabs of mineral wool and coconut fiber and then inserted into pots with perlite substrate. The pots were placed into a biosphere located 5 m below the sea level. The fertilizing solution (5% v/v of Aerogarden) was provided every 2 weeks. Basil plants were collected on 13 October 2015 and brought to the surface with the aid of pressurized cases. To avoid burning damages, they have been kept away from direct light prior to analyses. Inside the biosphere, only the natural lighting was exploited: maximum light intensity ranged between 8000 and 10000 lux (152-190 µmol/m^2/s) with natural photoperiod. The daily temperature ranged between 27 and 30 °C. The temperature variation between day and night was around 3-4 °C, with an average relative humidity around 80%. Samples were collected and used either fresh or dried at natural room conditions.

2.2. Phytochemical analyses

2.2.1. Essential oil hydrodistillations

The hydrodistillations were performed in a Clevenger type apparatus, equipped with an electric mantle heater for 2 hours (traditional method). The control sample extraction yield is 0.016% (calculated on 61 g fresh weight); the Nemo's Garden sample extraction yield is 0.025% (calculated on 80 g fresh weight).

2.2.2. *Head-Space Solid Phase Micro-Extraction Sampling*

Supelco SPME (Solid Phase Micro-Extraction) devices coated with polydimethylsiloxane (PDMS, 100 µm) were used to sampling the headspace. SPME sampling was performed using the same new fibre, preconditioned according to the manufacturer instructions, for all the analyses. Sampling was accomplished in an air-conditioned room (22±1°C) to guarantee a stable temperature. After 30 minutes of equilibration time, the fiber was exposed to the headspace for 2 minutes. Once sampling was finished, the fiber was

withdrawn into the needle and transferred to the injection port of the GC-MS system. The 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 peaks areas were performed between the same chemicals in the different samples.

2.2.3. *GC/MS and Volatiles Analysis*

The GC/EI-MS analyses were performed with a Varian CP-3800 apparatus equipped with a DB-5 capillary column (30 m X 0.25 mm i.d., film thickness 0.25 µm) and a Varian Saturn 2000 ion-trap mass detector. The oven temperature was programmed rising from 60° C to 240° C at 3° C/min; injector temperature, 220°C; transfer-line temperature, 240° C; carrier gas, He (1 mL/min).

The identification of the constituents was based on the comparison of their retention times (t_R) with those of pure reference samples and their linear retention indices (LRIs) determined relatively to the t_R of a series of *n*-alkanes. The mass spectra were compared with those listed in the commercial libraries NIST 14 and ADAMS and in a home-made mass-spectral library, built up from pure substances and components of known oils, and MS literature data (R. P. Adams, Zanoni, Lara, Barrero, & Cool, 1997; Robert P. Adams, 1995; Davies, 1990; Jennings & Shibamoto, 1982; Masada, 1976; Stenhagen, Abrahamsson, & McLafferty, 1974; Swigar & Silverstein, 1981).

2.3. Physiological analyses

2.3.1. Pigment analyses

Total chlorophyll and carotenoids contents were determined using the method described by Lichtenthaler (Lichtenthaler, 1987). Fresh leaves (50 mg fresh weight) were extracted in 5 mL of methanol and kept at 4°C in the dark for 24 h. The absorbance of the extracts at 665, 652, and 470 nm was measured using a UV-VIS spectrophotometer (Cintra 101, GBC Scientific Equipment LTD, Dandenong, Australia) and the content of total chlorophyll and carotenoids were expressed as mg g^{-1} fresh weight. The presented data are the means of three independent replicates.

145 *2.3.2. Total phenolic compounds*

146 Dried leaves (0.02 g) were pulverized and homogenized in a mortar with 1 mL of 70% (v/v) methanol to
147 facilitate the extraction. After 30 minutes of incubation on ice, the extracts were centrifuged at 14.000 *g* for
148 20 minutes at room temperature to collect the supernatant (methanol extract) to be used for the determination
149 of secondary metabolites. Total soluble polyphenolic compounds were assayed in different sample extracts
150 using the Folin-Ciocalteau's phenol protocol with minor modification (Singleton & Rossi, 1965). 0.5 mL of
151 Folin-Ciocalteau's reagent and 0.45 mL of sodium carbonate (7.5% w/v) were added to 1 mL of total volume
152 sample. After the incubation at room temperature for 2 h, the absorbance at 765 nm of the samples was
153 measured in UV-VIS spectrophotometer (Cintra 101, GBC Scientific Equipment LTD, Dandenong,
154 Australia) and referred to a standard curve for gallic acid prepared in the range of 0-50 mg/mL. All
155 determinations were performed in triplicate.

156 *2.3.3. DPPH scavenging ability*

157 The antioxidant activity of each basil methanol extract was determined using a modified version of the 2,2-
158 diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay (Kim, Chun, Kim, Moon, & Lee, 2003). The
159 activity was measured as a decrease in absorbance at 517 nm using the UV-VIS spectrophotometer. The
160 percent inhibition of the DPPH radical by the samples was calculated according to the formula:

161 $\%\ \text{inhibition} = (A_{blank}-A_{sample} / A_{blank}) \times 100$

162 where A_{blank} is the absorbance of the DPPH and A_{sample} is the absorbance of the samples. The extract
163 concentration (µg/mL) providing 50% of antioxidant activities (IC_{50}) was calculated by plotting on a graph
164 inhibition percentage against extract concentration. All determinations were performed in triplicate.

165 *2.4. Micromorphological analyses*

166 Fresh mature leaves for micromorphological investigation were gathered simultaneously to the collection of
167 the plant material for both phytochemical and physiological analyses. At least ten leaves, similar for total
168 size, position and developmental stage were selected from the control and Nemo's plants.

169 Light microscopy (LM) and scanning and transmission electron microscopy (SEM and TEM) were used to
170 examine the different types of secreting trichomes, their distribution pattern, their histochemistry and the
171 ultrastructure of the glandular cells.

172 *2.4.1. SEM investigation*

173 Plant material was first hand-prepared, fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2),
174 dehydrated in an ascending ethanol series up to absolute and then dried using a critical-point-dryer apparatus.
175 The samples, mounted on aluminum stubs, were coated with gold and observed with a Philips XL 20 SEM
176 operating at 10 kV.

177 *2.4.2. LM investigation*

178 The samples were frozen, sectioned and stained with various histochemical techniques in order to evidence
179 the chemical nature of the secretory products and to specifically locate the sites of terpene accumulation and
180 release. The following methods were employed: Fluoral Yellow 088 for total lipids (Brundrett et al., 1991),
181 Nile Red for neutral lipids (Greenspan et al., 1985), Nadi reagent for terpenes (David and Carde, 1964),
182 Ruthenium Red (Jensen, 1962) and Alcian Blue (Beccari and Mazzi, 1966) for acidic polysaccharides,
183 Mercuric Bromophenol Blue for proteins (Mazia et al., 1953), Ferric Trichloride for polyphenols (Gahan,
184 1984) and Aluminium Trichloride for flavonoids (Mazia et al., 1953). Control procedures were carried out at
185 the same time. Observations were made with a Leitz DM-RB Fluo optical microscope.

186 *2.4.3. TEM investigation*

187 Small segments of plant material were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.2 and
188 post-fixed in 2% OsO_4, dehydrated in ethanol in ascending grades up to absolute, and embedded in Spurr's
189 resin. Ultrathin sections were stained with uranile acetate and lead citrate. Samples were examined with a
190 Philips EM-300 TEM.

191 **3. Results and discussion**

192 *3.1. Phytochemical investigation*

193 *3.1.1. Essential oils compositions*

194 The control basil extraction yield is 0.016%. This essential oil composition reveals a methyl eugenol –
195 linalool chemotype: the former is a phenylpropanoid, which accounts for 22.8% (see Table 2), whilst the
196 latter is an oxygenated monoterpene with a relative abundance of 20.1%. These two classes of compounds
197 are the most relevant ones: combined, they represent more than 60% of the total composition, as each one
198 accounts for more than 30%. Eugenol, another phenylpropanoid, follows, with a relative abundance of 8.7%.
199 Among oxygenated monoterpenes, 1,8-cineole shows a relevant presence (6.9%). Sesquiterpene
200 hydrocarbons are the third most represented chemical class (24.8%), of which *trans*-α-bergamotene is the
201 most abundant one (7.4%).

202 Basil shows a wide number of chemotypes: cultivars and the geographical origin are the main reasons for
203 such a variability in its essential oil composition. Lawrence (1988) identified four main chemotypes of basil:
204 i) methyl chavicol, ii) linalool, iii) methyl eugenol, iv) methyl cinnamate. Grayer et al., 2004 reported that
205 basil essential oil major (relative abundance over 20%) components are extremely variable among different
206 genotypes: these compounds are generally methyl chavicol, eugenol and/or methyl eugenol among the
207 phenylpropanoids and/or linalool, geraniol, geranial and/or neral among the oxygenated monoterpenes.
208 Grayer et al. (1996) classified 5 main basil chemotypes: i) linalool, ii) methyl chavicol, iii) linalool and
209 methyl chavicol, iv) linalool and eugenol, v) methyl chavicol and methyl eugenol. Koutsos et al. (2009)
210 identified four main basil chemotypes based on their geographical origin: i) European basil, rich in linalool
211 (35-50%) and methyl chavicol (15-25%); ii) Reunion basil, rich in methyl chavicol (>80%); iii) Tropical
212 basil, rich in methyl cinnamate; iv) Java basil, rich in eugenol. Özcan and Chalchat (2002) reported the
213 composition of a Turkish *Ocimum basilicum* mainly rich in methyl eugenol: in their essential oil, it accounts
214 for up to 78.02%. The relevant presence of methyl eugenol in basil essential oil has been studied by Miele et
215 al. (2001): they reported a negative correlation on plants height and methyl eugenol relative abundance, since
216 in young specimens (up to 10-12 cm) methyl eugenol showed a more relevant presence than in older (and
217 taller) ones. In this study, both control and Nemo's Garden® samples are young specimens, approximately 7
218 cm high: as well as a chemotype matter, this could be the reason of such a relevant methyl eugenol relative
219 abundance.

220 The composition of Nemo's Garden® basil essential oil (extraction yield 0.025%) shows a methyl eugenol
221 chemotype: this phenylpropanoid represents 49.6% (see Table 2) of the essential oil. The very same species
222 of basil grown in different environmental conditions shows a shift of chemotype: from methyl eugenol –
223 linalool to methyl eugenol, as linalool only accounts for 1.3%. In comparison with the control sample,
224 eugenol relative abundance is more than doubled. Moreover, differently from the control sample,
225 sesquiterpene hydrocarbons are now the second most abundant (19.1%) chemical class of compounds:
226 among these, α-humulene (5.9%) and *trans*-α-bergamotene (4.7%) have the largest abundance.

227 The shading conditions in the biospheres are heavy, as the light intensity that reaches the plants is reduced by
228 80-90% (152-190 μmol/m^2s) in comparison with unshaded greenhouse conditions (600-1600 μmol/m^2s on
229 average (Chang et al. (2008))) due to the water depth and the biosphere material. Chang et al. (2008) studied
230 the behaviour of the three major compounds (eugenol, methyl eugenol and linalool) in the essential oils
231 hydrodistilled from *O. basilicum* cv. 'Basil Sweet Genovese' grown under different shading conditions
232 obtained with shading nets. High daily light integrals significantly increased linalool and eugenol relative
233 abundances, whilst methyl eugenol showed a relevant increment with lower daily light integrals. Other
234 aroma active compounds of basil, like 1,8-cineole, weren't influenced by the intensity of light. In accordance
235 with the latter, in the present study, linalool shows a significant decrement from the control (20.1%) to the
236 Nemo's Garden® (1.3%) sample and methyl eugenol evidenced a more than two-fold enhancement (from
237 22.8% in the control conditions to 49.6% in the Nemo's biospheres). However, in the studied basil, eugenol
238 shows an increment from control (8.7%) to underwater biosphere (17.2%) conditions: this could be due to
239 the differences in the red/far-red ratio, as the light quality is as important a parameter as the light intensity
240 (Morelli & Ruberti, 2002). The total amount of essential oil significantly increased with increase of the
241 radiant energy, particularly detected in the case of the most important flavor compounds 1,8-cineole,
242 linalool, and eugenol. Moreover, the level of the main compound, methyleugenol, significantly decreased
243 with UV-B radiation: this result is important because this compound is of toxicological concern to human
244 health due to the structural similarity to known carcinogenic phenylpropanoids, such as estragole (Nitz &
245 Schnitzler, 2004).

246 *3.1.2. Head-Space Solid Phase Micro-Extraction (HS-SPME)*

247 The spontaneous volatile emission of the aerial parts of the control sample is mainly rich in monoterpenes,
248 that cumulatively reach 74.2% of the total head-space. The oxygenated ones represent half of the total
249 emission (51.6%, see Table 3): 1,8-cineole is the most abundant VOC, accounting for 43.1%, followed by
250 linalool (7.2%). The most important monoterpene hydrocarbons are β-pinene (6.8%) and (*E*)-β-ocimene
251 (6.5%). Among sesquiterpene hydrocarbons, *trans*-α-bergamotene is the most abundant one (7.3%).

252 In the basil head-space from Nemo's Garden®, the aroma profile is dominated by sesquiterpene
253 hydrocarbons, that reach up to 61.2%. Among these, *trans*-α-bergamotene is the most relevant (26.0%),
254 followed by α-humulene (17.3%). Oxygenated monoterpenes are significantly represented (27.2%) in this
255 sample head-space, as well, and 1,8-cineole alone represents most of this class relative abundance, as it
256 accounts for 25.4%. Phenylpropanoids show a similar relevance in both samples: 4.7 and 4.5% in the control
257 and Nemo's Garden® basil, respectively.

258 (*E*)-β-farnesene is significantly represented in the control sample, where it accounts for up to 6.0%, whilst it
259 is not detected in the biosphere sample. The opposite behaviour is shown by γ-muurolene: it is not detected
260 in the control sample, while it reaches 4.5% in the Nemo's basil head-space.

261 These divergent emission profiles are due to the different growth environment conditions. Besides the
262 metabolic changes induced by the different light and humidity, the biospheres represent a closed and
263 protected environment, in which no pollinators, nor parasites, are present.

264 *3.2. Physiological investigation: metabolites analyses*

265 The results are reported in Table 4. The analysis of photosynthetic pigments (chlorophylls and carotenoids)
266 showed that basil plants grown in Nemo's Garden® have higher amounts of these compounds than control
267 plants. This can be due to the lower level of irradiance of Nemo's plants, so that the photosynthetic pigments
268 are more present to counteract the low efficiency. In fact, the content of Chlorophyll a (Chla), the most
269 important for the photons capture, is in good balance with the amount of Chlorophyll b (Chlb) in control
270 plants, whereas Nemo's plants showed a lower content of Chla: therefore, the Chla/Chlb ratio is optimal for
271 control plants, but very low for Nemo's plants. The carotenoids/chlorophylls ratio may often be a good
272 indicator of stress in plants (Hendry & Price, 1993): in Nemo's basil the ratio is similar to that of the control

one, indicating that the plants are well adapted to this new condition. To better analyze the avoidance of some stress conditions, total polyphenols have been determined. Polyphenols content is slightly higher in Nemo's plants than in control leaves: according to these results, the antioxidant activity (expressed as IC_{50} of DPPH antioxidant activity) is lower than in control plants. In past papers, Shiga et al. (2009) demonstrated that basil leaves were influenced by light treatments, changing their relative polyphenol content and the corresponding antioxidant activity. Cheynier et al. (2013) reported that the polyphenols synthesis is influenced (stimulated, in some cases) by exposure to a specific light spectrum. On the other hand, the mechanism of the influence is genus- or species-specific, therefore the overall mechanism is not well understood. Demotes-Mainard et al. (2016) described the influence of red and far red light on the vegetative and reproductive stages of horticultural plants. However, they concluded that the phenotypic response to red, far-red and R:FR can vary among species, but also with growing conditions. Studies aiming at the discovery of the mechanisms of such differences can include the plants of Nemo's Garden®, as well. The results of the metabolites detected in Nemo's plant indicate that the plants do not show oxidative stress, although some light influence should be better investigated.

3.3. Micromorphological investigation

3.3.1. SEM investigation

SEM observations allowed to examine and compare trichome morphotypes and distribution on the leaves of both control and Nemo's samples. A high level of consistency was found for the *indumentum* features (Fig. 1a-e).

The non-glandular hairs are short, simple, uniseriate, with a pointed apex and a smooth cuticular surface; they are predominantly located on the median and secondary ribs of the abaxial leaf surface (Fig. 1a-c). As regards to the glandular trichomes, peltates and two basic types of capitates have been observed (Fig. 1).

The peltates are constituted by one or two basal epidermal cells, one neck cell and by a four-celled secreting head (40-60 μm in diameter, Fig. 1d-e), surmounted by a wide subcuticular space where the secreted material accumulates; the breakage of the outer cuticle is occasionally observed (Fig. 1c). The capitate trichomes are formed by a basal epidermal cell, one neck cell and by one or two apical secreting cells. The

299 diameter of the glandular head is about 20-25 µm, while the trichome length is approximately 30 µm (Fig. 1
300 d-e) The diversity in head morphology allowed the recognition of two types of capitate hairs: type I with a
301 unicellular head and type II with a bicellular head (Fig. 1 d-e), the first being sporadic.

302 The leaf primordia show a high density of glandular trichomes at the proximal and middle regions, while the
303 distal portion appears mostly hairless. With the ongoing of leaf development, trichomes density decreases.
304 The adaxial and abaxial surfaces exhibit a homogeneous distribution pattern (Fig. 1a-b): capitates are
305 preferably located along the veinal system, whereas peltates are uniformly distributed over the entire lamina.

306 These trichomes possess overall morphological features comparable to those already known in the literature
307 (Giuliani and Maleci Bini, 2008; Hallahan, 2000; Werker, 2000).

308 *3.3.2. LM investigation*

309 The results of the histochemical investigation are reported in Table 5. The chemical nature of the secretory
310 products of all the glandular trichomes proved consistent in the control and Nemo's plants.

311 The peltates exhibit great affinity for the dyes specific for lipophilic substances: indeed, intense orange and
312 yellow-greenish colorations of the secretory products result following the application of Nile Red and
313 Fluoral Yellow-088, respectively. The NADI reagent, specific for terpenes, displays a strong positive
314 response (Fig. 1f). The dyes for total phenols and flavonoids evidence the cytoplasm of the secreting cells.

315 Type I capitate trichomes show an exclusive positive response to the NADI reagent, which highlights the
316 glandular head and few droplets of secreted material outside the apical periclinal wall (Fig. 1g). The secreted
317 material of type II capitates shows affinity only for the dyes specific for polysaccharides (PAS reaction, Fig.
318 1h) and proteins.

319 The peltates and type I capitates are typical terpene producers, whereas the type II capitates are responsible
320 for the synthesis of polysaccharides. Minor fractions of polyphenols and flavonoids, beside the dominance of
321 terpenes, are presumably produced by peltates, but a clear response is not achieved for these types of
322 substances.

323 Based on these observations, the overall production of volatiles and essential oils is related to the activity of
324 peltates and type I capitate.

325 *3.3.3. TEM investigation*

326 TEM observations involve the secreting cells of mature peltate and of type II capitate trichomes (Fig. 1 i-k).
327 They allowed to confirm the preliminary histochemical results.

328 In all the types of glandular hairs numerous plasmodesmata cross the periclinal walls between all the cells
329 constituting the trichome and the anticlinal walls of the secreting head. This ultrastructural feature evidences
330 that all the trichome cells are involved in the production and release of the secreted material.

331 In the active peltate trichomes, the most striking ultrastructural feature is the occurrence of numerous plastids
332 with an irregular internal membrane system and evident plastoglobuli associated to periplastidial smooth
333 endoplasmic reticulum (Fig. 1i). In the area below the subcuticular space the plasmalemma is crenulated and
334 slightly detached from the wall, forming a thin periplasmatic space in which small vesicles are visible (Fig.
335 1j). At this stage, the well-developed subcuticular space contains materials of different appearance: small
336 electrondense globules of lipophilic nature, immersed in an abundant granular matrix, presumably
337 constituted by phenols. These evidences confirm the results of the histochemical tests as abundant plastids
338 and smooth endoplasmic reticulum are the cell compartments responsible for the production and transport of
339 terpenic substances, which are among the main components of the essential oil (Hallahan, 2000).

340 At the active secretory phase, the secreting cell cytoplasm of the type II trichomes is characterized by
341 abundant dictyosomes, originating a large number of vesicles, and by a well-developed rough endoplasmic
342 reticulum often surrounding vacuoles (Fig. 1k). These ultrastructural features, and the histochemical results
343 of the PAS reaction, indicate the production of polysaccharides (Giuliani and Maleci Bini, 2008). In addition,
344 the occurrence of rough endoplasmic reticulum in association with dictyosomes suggests that the
345 polysaccharidic secretion is associated with the synthesis of proteic material.

346 **4. Conclusions**

347 The most evident phytochemical responses to the growth conditions the samples have undergone inside the
348 biospheres were the essential oil chemotype switch and the very different spontaneous emission patterns,

highlighting the plant fast response to the new habitat. The differences in the spontaneously emitted volatiles were more apparent than those in the essential oils if compared with the control plants: this was most probably due to the differences in the environment, including the absence of pollinators, competing plants and parasites, since the biosphere is a closed underwater space. The irradiance of basil cultivated in Nemo's Garden® biospheres, under several meters of seawater, lead to a change in the level of photosynthetic pigments, although no micromorphological changes of the leaf *indumentum* were evidenced. It may, therefore, be stated that the plants are well adapted to survive and grow in such conditions, as the occurred changes in polyphenols amounts and antioxidant activity are less pronounced.

The biospheres environment, thus, affected more the phytochemical and physiological responses: the chemotype change and very different volatile profiles were evidenced for the former, and an increased production of chlorophylls, carotenoids and polyphenols for the latter. The micromorphology of the plants, though, was not affected.

The Nemo's Garden® underwater farm represents a promising alternative system to standard agriculture to be introduced in areas where the cultivable soil is scarce, or the climatic conditions are not good for some type of plants. Indeed, the underwater farm provides a new environment for plants to grow in. Further studies are needed to assess the adaptation of distinct species to the marine conditions, especially pressure. This pioneering plant growth system could be interestingly applied to grow food and/or spice plants, as well as being adapted for species of pharmaceutical interest, whose useful secondary metabolites could increment/change in a desirable direction due to the various stress conditions they are subjected to.

Acknowledgements

The authors would like to thank Sergio Gamberini, Luca Gamberini, Gabriele Cucchia and Dario Piombo from Ocean Reef Group for their technical support.

Conflict of interest

The authors have no conflict of interest to declare.

References

Adams, R. P. (1995). *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy*. Carol Stream, Illinois, USA: Allured Publishing Corporation.

Adams, R. P., Zanoni, T. A., Lara, A., Barrero, A. F., & Cool, L. G. (1997). Comparisons among *Cupressus arizonica* Greene, *C. benthamii* Endl., *C. lindleyi* Klotz, ex Endl. and *C. lusitanica* Mill, using Leaf Essential Oils and DNA Fingerprinting. *Journal of Essential Oil Research*, *9*(3), 303–309. https://doi.org/10.1080/10412905.1997.10554249

Beccari, N., & Mazzi, V. (1966). *Manuale di tecnica microscopica*. Como, Italy: Soc. Editrice Libraria.

Brundrett, M. C., Kendrick, B., & Peterson, C. A. (1991). Efficient lipid staining in plant material with sudan red 7B or fluorol [correction of fluoral] yellow 088 in polyethylene glycol-glycerol. *Biotechnic & hHstochemistry : Official Publication of the Biological Stain Commission*, *66*(3), 111–116. https://doi.org/10.3109/10520299109110562

Chang, X., Alderson, P. G., & Wright, C. J. (2008). Solar irradiance level alters the growth of basil (Ocimum basilicum L.) and its content of volatile oils. *Environmental and Experimental Botany*, *63*(1–3), 216–223. https://doi.org/10.1016/j.envexpbot.2007.10.017

Cheynier, V., Comte, G., Davies, K. M., Lattanzio, V., & Martens, S. (2013). Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry*, *72*, 1–20. https://doi.org/10.1016/j.plaphy.2013.05.009

David, R., & Carde, J. P. (1964). Coloration différentielle des inclusions lipidiques et terpéniques des pseudophylles du Pin maritime au moyen du réactif NADI. *Comptes Rendus de I'Academie Des Sciences Paris*, *258*, 1338–1340.

Davies, N. W. (1990). Gas chromatographic retention indices of monoterpenes and sesquiterpenes on Methyl Silicon and Carbowax 20M phases. *Journal of Chromatography A*, *503*, 1–24.

Demotes-Mainard, S., Péron, T., Corot, A., Bertheloot, J., Le Gourrierec, J., Pelleschi-Travier, S., … Sakr, S. (2016). Plant responses to red and far-red lights, applications in horticulture. *Environmental and Experimental Botany*, *121*, 4–21. https://doi.org/10.1016/j.envexpbot.2015.05.010

Dini, G., Princi, E., Gamberini, S., & Gamberini, L. (2016). Nemo's Garden: growing plants underwater. In *OCEANS'16 MTS/IEEE*. Monterey, USA.

Gahan, P. B. (1984). *Plant histochemistry and cytochemistry: an introduction*. London: Academic Press London.

Giuliani, C., & Maleci Bini, L. (2008). Insight into the structure and chemistry of glandular trichomes of Labiatae, with emphasis on subfamily Lamioideae. *Plant Systematics and Evolution*, *276*, 199–208. https://doi.org/10.1007/s00606-008-0085-0

Grayer, R. J., Vieira, R. F., Price, A. M., Kite, G. C., Simon, J. E., & Paton, A. J. (2004). Characterization of cultivars within species of Ocimum by exudate flavonoid profiles. *Biochemical Systematics and Ecology*, *32*(10), 901–913. https://doi.org/10.1016/j.bse.2004.04.002

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *The Journal of Cell Biology*, *100*(10), 965–973. https://doi.org/10.1083/jcb.100.3.965

Hallahan, D. L. (2000). Monoterpenoid biosynthesis in glandular trichomes of Labiatae plants. In D. L. Hallahan & J. C. Gray (Eds.), *Advances in Botanical Research: Plant Trichomes* (pp. 77–120). New York - London: Academic Press.

Hendry, G. A. F., & Price, A. H. (1993). Stress indicators: chlorophylls and carotenoids. In G. A. F. Hendry & J. P. Grime (Eds.), *Methods in Comparative Plant Ecology* (1st ed., pp. 148–152). Springer Netherlands. https://doi.org/10.1007/978-94-011-1494-3

Hussain, A. I., Anwar, F., Hussain Sherazi, S. T., & Przybylski, R. (2008). Chemical composition, antioxidant and antimicrobial activities of basil (Ocimum basilicum) essential oils depends on seasonal variations. *Food Chemistry*, *108*(3), 986–995. https://doi.org/10.1016/j.foodchem.2007.12.010

INTRACEN, M. I. (2016). *INTRACEN Essential Oils and Oleoresins Market Insider August 2016 Report*.

Jennings, W., & Shibamoto, T. (1982). *Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography*. *Food / Nahrung* (Vol. 26). New York, London, Sydney, Toronto, San Francisco: Academic Press.

Jensen, W. A. (1962). *Botanical histochemistry: principles and practice*. San Francisco: WH Freeman & Co.

Kim, D.-O., Chun, O. K., Kim, Y. J., Moon, H.-Y., & Lee, C. Y. (2003). Quantification of Polyphenolics and Their Antioxidant Capacity in Fresh Plums. *Journal of Agricultural and Food Chemistry*, *51*(22), 6509–6515. https://doi.org/10.1021/jf0343074

Koutsos, T. V., Chatzopoulou, P. S., & Katsiotis, S. T. (2009). Effects of individual selection on agronomical and morphological traits and essential oil of a "Greek basil" population. *Euphytica*, *170*(3), 365–370. https://doi.org/10.1007/s10681-009-0012-7

Lawrence, B. M. (1988). A further examination of the variation of Ocimum basilicum L. in flavors and fragrances: a world perspective. In B. M. Lawrence, B. M. Mookherjee, & B. J. Willis (Eds.), *Proceedings of the 10th International Congress of Essential Oils, Fragrance and Flavors* (pp. 161–170). Washington D.C. (USA): Elsevier Sci. Publishers B. U.

Lee, S.-J., Umano, K., Shibamoto, T., & Lee, K.-G. (2005). Identification of volatile components in basil (Ocimum basilicum L.) and thyme leaves (Thymus vulgaris L.) and their antioxidant properties. *Food Chemistry*, *91*(1), 131–137. https://doi.org/10.1016/j.foodchem.2004.05.056

Lichtenthaler, H. K. (1987). [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In *Methods in Enzymology* (pp. 350–382). Elsevier. https://doi.org/10.1016/0076-6879(87)48036-1

Masada, Y. (1976). *Analysis of essential oils by gas chromatography and mass spectrometry*. New York, NY: John Wiley & Sons, Inc.

Mazia, D., Brewer, P. A., & Alfert, M. (1953). The cytochemical staining and measurement of protein with mercuric bromphenol blue. *The Biological Bulletin*, *104*(1), 57–67.

Miele, M., Ledda, B., Falugi, C., & Mazzei, M. (2001). Methyleugenol and eugenol variation in Ocimum basilicum cv. Genovese gigante grown in greenhouse and in vitro. *Boll Soc Ital Biol Sper*, *77*(4–6), 43–50.

Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11822201

Morelli, G., & Ruberti, I. (2002). Light and shade in the photocontrol of Arabidopsis growth. *Trends in Plant Science*, *7*(9), 399–404. https://doi.org/10.1016/S1360-1385(02)02314-2

Nitz, G. M., & Schnitzler, W. H. (2004). Effect Of PAR And UV-B Radiation on the Quality and Quantity of the Essential Oil in Sweet Basil (Ocimum Basilicum L.). *Acta Horticulturae*, (659), 375–381. https://doi.org/10.17660/ActaHortic.2004.659.50

Özcan, M., Arslan, D., & Ünver, A. (2005). Effect of drying methods on the mineral content of basil (Ocimum basilicum L.). *Journal of Food Engineering*, *69*(3), 375–379. https://doi.org/10.1016/j.jfoodeng.2004.08.030

Özcan, M., & Chalchat, J.-C. (2002). Essential Oil Composition of Ocimum basilicum L. and Ocimum minimum L. in Turkey. *Czech Journal Of Food Sciences*, *20*(6), 223–228. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=14483079

Politeo, O., Jukic, M., & Milos, M. (2007). Chemical composition and antioxidant capacity of free volatile aglycones from basil (Ocimum basilicum L.) compared with its essential oil. *Food Chemistry*, *101*(1), 379–385. https://doi.org/10.1016/j.foodchem.2006.01.045

Princi, E., Gamberini, L., Gamberini, S., Cucchia, G., Fontanesi, G., & Piombo, D. (2016). Nemo's Garden: growing plants underwater. In *MELiSSA Worshop*. Lausanne: European Space Agency.

Sgherri, C., Cecconami, S., Pinzino, C., Navari-Izzo, F., & Izzo, R. (2010). Levels of antioxidants and nutraceuticals in basil grown in hydroponics and soil. *Food Chemistry*, *123*(2), 416–422. https://doi.org/10.1016/j.foodchem.2010.04.058

Shiga, T., Shoji, K., Shimada, H., Hashida, S., Goto, F., & Yoshihara, T. (2009). Effect of light quality on rosmarinic acid content and antioxidant activity of sweet basil, Ocimum basilicum L. *Plant Tissue Culture Letters*, *26*(2), 255–259. https://doi.org/10.5511/plantbiotechnology.26.255

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158. Retrieved from http://www.ajevonline.org/content/16/3/144

Stenhagen, E., Abrahamsson, S., & McLafferty, F. W. (1974). *Registry of Mass spectral data*. New York, NY: Wiley & Sons.

Swigar, A. A., & Silverstein, R. M. (1981). *Monoterpenes*. *Aldrich Chemical Company*. Milwaukee, Wisconsin: Aldrich Chemical Company.

United Nations Industrial Development Organization and Food and Agriculture Organization. (2005). *Herbs , spices and essential oils. Post-harvest operations in developing countries*. *Unido and Fao*.

Werker, E. (2000). Trichome diversity and development. In D. L. Hallahan & J. C. Gray (Eds.), *Advances in Botanical Research: Plant Trichomes* (pp. 1–35). New York - London: Academic Press.

Caption to Figure 1

a-c. Trichomes distribution pattern in the Nemo's samples of *Ocimum basilicum*, SEM: a. Leaf abaxial surface with glandular and non-glandular trichomes. b. Leaf adaxial surface with glandular and non-glandular trichomes c. Particular of the leaf abaxial surface. d-e. Trichomes morphotypes in *Ocimum basilicum* - peltates, type I and II capitates, SEM: Nemo's samples (d); control samples (e). f-h. Histochemistry of the glandular trichomes of *Ocimum basilicum*, LM: Nadi reagent in peltate (f) and capitate type I (g) trichome; PAS reaction in type II trichome (h). i-k. Ultrastructure of the glandular trichomes of *Ocimum basilicum*, TEM: secreting cell cytoplasm of a peltate trichome (i); particular of the outer anticlinal wall in a peltate trichome (j); secreting cell cytoplasm of a type II capitate trichome (K).

Symbols: cu, cuticular layer; D, dictyosomes; p, plastid; pc, pectic-celluosic layer; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; v, vacuole

Tables

Table 1 INTRACEN Essential Oils and Oleoresins October 2016 Report on basil EO

Production method	**Origin**	**Prices ($) / kg**
Standard	Comoros	125
	Egypt	82
	Vietnam	90
	India	15
Organic	Egypt	187

501 **Table 2** Compositions of the essential oils hydrodistilled from the control sample and the Nemo's Garden

502 basil

l.r.i.[a]	Constituents	Relative abundance (%)	
		CONTROL	NEMO'S
928	tricyclene	0.2	-[b]
941	α-pinene	-	0.1
976	sabinene	0.4	0.2
982	β-pinene	0.7	0.3
993	myrcene	0.8	0.2
1001	octanal	0.1	tr[c]
1011	δ-3-carene	0.1	tr
1034	1,8-cineole	6.9	4.8
1052	(*E*)-β-ocimene	1.3	0.6
1070	*cis*-sabinene hydrate	0.3	0.2
1088	terpinolene	0.8	0.3
1101	linalool	20.1	1.3
1111	1-octen-3-yl acetate	0.2	-
1143	camphor	0.6	0.7
1170	δ-terpineol	0.1	0.1
1191	α-terpineol	0.8	0.6
1197	methyl chavicol	-	0.8
1214	*n*-octanol acetate	0.4	0.2
1285	*iso*bornyl acetate	2.8	1.0
1352	α-terpinyl acetate	0.2	-
1358	eugenol	8.7	17.2
1376	α-copaene	0.2	-
1380	(*E*)-methyl cinnamate	-	0.7
1390	β-cubebene	0.6	-
1392	β-elemene	0.2	0.2
1403	methyl eugenol	22.8	49.6
1420	β-caryophyllene	0.2	0.9
1438	*trans*-α-bergamotene	7.4	4.7
1456	α-humulene	1.6	5.9
1460	(*E*)-β-farnesene	5.2	3.1
1462	*cis*-muurola-4(14),5-diene	0.1	-
1477	γ-muurolene	-	2.0
1481	germacrene D	2.7	-
1490	(*E*,*Z*)-α-farnesene	0.7	-
1495	bicyclogermacrene	1.9	0.5
1505	α-bulnesene	2.3	1.3
1513	*trans*-γ-cadinene	1.3	0.2
1524	β-sesquiphellandrene	0.5	0.3
1535	(*E*)-γ-bisabolene	-	0.1
1581	caryophyllene oxide	0.2	-
1614	1,10-*di*-*epi*-cubenol	0.6	tr

1640	*epi*-α-cadinol	6.0	0.5
1650	β-eudesmol	-	0.5
1692	methyl-*p*-methoxycinnamate	-	0.1
1843	(*E*,*E*)-farnesyl acetate	-	0.6
	Monoterpene hydrocarbons	4.2	1.7
	Oxygenated monoterpenes	31.8	8.7
	Sesquiterpene hydrocarbons	24.8	19.1
	Oxygenated sesquiterpenes	6.8	1.6
	Phenylpropanoids	31.4	68.4
	Other non-terpene derivatives	0.8	0.2
	Total identified (%):	99.7	99.6
	Extraction yield (% w/w):	0.016	0.025

[a] Linear retention indices on a DB5 column
[b] Not detected
[c] Traces, relative abundance <0.1%

503

504 **Table 3** Volatile organic compounds in the samples head-spaces

l.r.i.[a]	Constituents	Relative abundance (%)	
		CONTROL	**NEMO'S**
982	β-pinene	6.8	3.6
993	myrcene	4.3	-[b]
1011	δ-3-carene	0.9	-
1034	1,8-cineole	43.1	25.4
1052	(*E*)-β-ocimene	6.5	0.9
1070	*cis*-sabinene hydrate	0.1	0.4
1088	terpinolene	3.0	1.6
1101	linalool	7.2	0.3
1143	camphor	0.8	0.7
1191	α-terpineol	0.4	0.5
1340	δ-elemene	0.2	0.6
1351	α-cubebene	0.1	-
1358	eugenol	0.8	1.9
1376	α-copaene	0.2	0.4
1390	β-cubebene	0.1	-
1392	β-elemene	1.0	3.9
1403	methyl eugenol	3.8	2.5
1416	*cis*-α-bergamotene	-	0.4
1420	β-caryophyllene	0.5	2.3
1438	*trans*-α-bergamotene	7.3	26.0
1441	aromadendrene	-	0.2
1456	α-humulene	0.9	17.3
1460	(*E*)-β-farnesene	6.0	-
1462	*cis*-muurola-4(14),5-diene	-	0.3
1477	γ-muurolene	-	4.5
1481	germacrene D	1.3	-
1490	(*E*,*Z*)-α-farnesene	0.4	-
1495	bicyclogermacrene	0.6	1.4
1505	α-bulnesene	0.6	2.4
1509	β-bisabolene	0.1	-
1513	*trans*-γ-cadinene	0.6	1.0
1524	β-sesquiphellandrene	0.3	0.6
1640	*epi*-α-cadinol	tr[c]	0.3
1815	2-ethylhexyl salicylate	0.4	-
1903	3,3,5-trimethylcyclohexyl salicylate	0.4	-
2000	*n*-eicosane	-	0.2
	Monoterpene hydrocarbons	22.6	6.1
	Oxygenated monoterpenes	51.6	27.2
	Sesquiterpene hydrocarbons	20.2	61.2
	Oxygenated sesquiterpenes	-	0.3
	Phenylpropanoids	4.7	4.5

	Other non-terpene derivatives	0.8	0.2
	Total identified:	99.8	99.4

[a] Linear retention indices on a DB5 column
[b] Not detected
[c] Traces, relative abundance <0.1%

505

506 **Table 4** Determination of foliar pigments (clorophyll a, chlorophyll b, total chlorophylls) and total
507 carotenoids (mg g−1 FW), polyphenol content (mg/g DW GA equivalent), IC_{50} of the free radical (DPPH)
508 scavenging activity of one-month old basil plants collected in Nemo's Garden® (September) and in control
509 plants grown in terrestrial aerial condition. Mean values were obtained from 3 independent replicates ± SD.

	***Ocimum basilicum* L. Nemo's Garden®**	***Ocimum basilicum.* L. Control**
Chlorophyll a (mg/g FW)	2.378 ± 0.006	0.942 ± 0.003
Chlorophyll b (mg/g FW)	2.156 ± 0.005	0.358 ± 0.001
Total Chlorophyll (mg/g FW)	4.534 ± 0.011	1.30 ± 0.004
Ratio Chlorophyll a/ Chlorophyll b	1.1	2.63
Total carotenoids (mg/g FW)	0.165 ± 0.01	0.065 ± 0.009
Ratio Carotenoids/ Chlorophylls	27.2	20
Total polyphenols (mg/g DW)	4.25 ± 0.15	3.75 ± 0.47
IC_{50} DPPH (mg DW/ml)	0.165 ± 0.05	0.217 ± 0.06

510

511 **Table 5** Histochemical results on the leaf glandular trichomes of the control and Nemo's plants of *Ocimum*

512 *basilicum*.

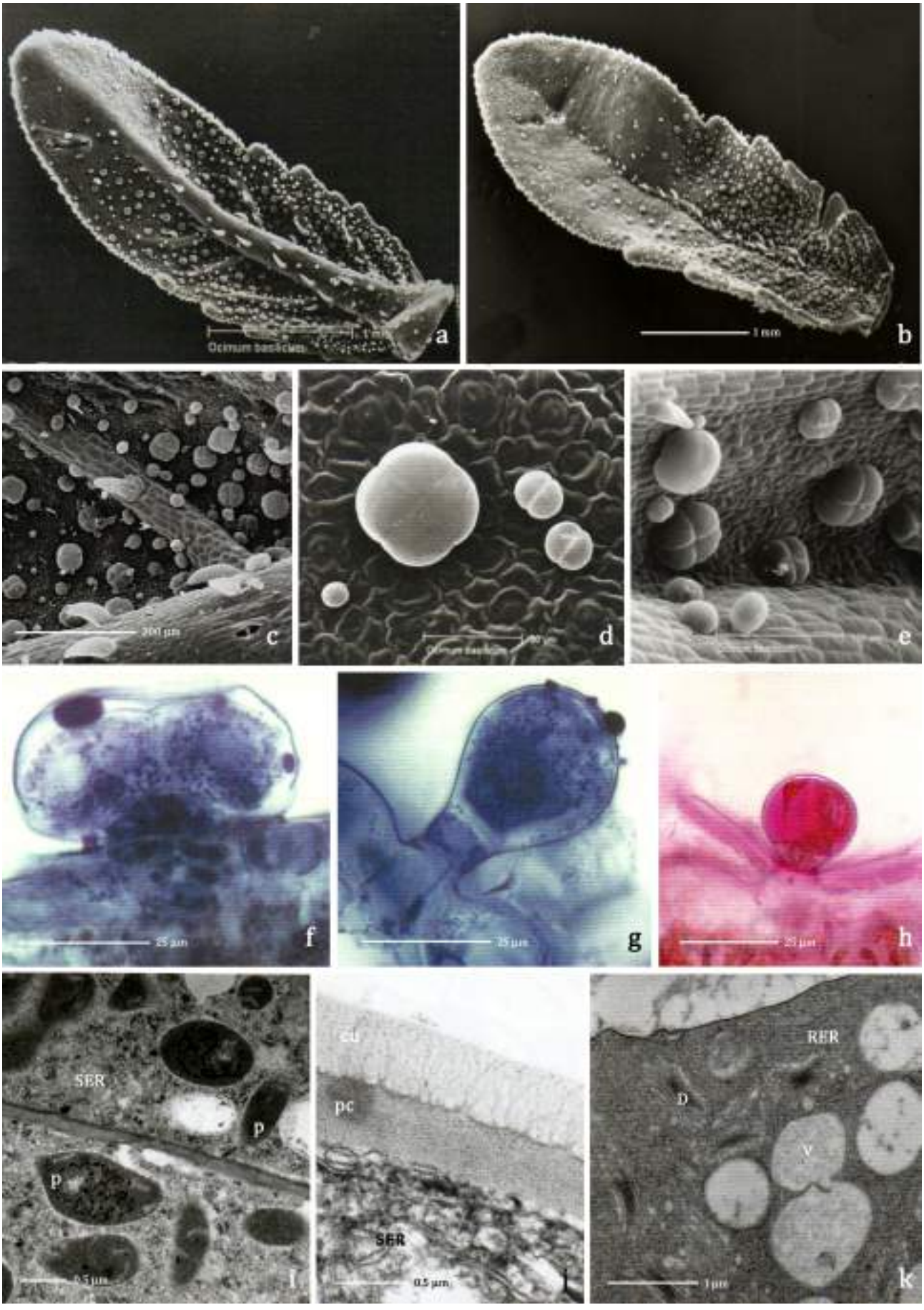
Staining procedure	Target compounds	Observed colour	peltate		type I capitate		type II capitate	
			Control	Nemo's	Control	Nemo's	Control	Nemo's
Nile red	Neutral lipids	Golden-yellow	++	++	-	-	-	-
Fluoral yellow-088	Total lipids	Yellow to orange	++	++	±	±	-	-
Nadi reagent	Terpenes	Violet-blue	++	++	+	+	-	-
$FeCl_3$	Polyphenols	Emerald-green	+ *	+ *	-	-	-	-
$AlCl_3$	Flavonoids	Blue-green	+ *	+ *	-	-	-	-
PAS reaction	Polysaccharides	Red-pinkish	-	-	-	-	++	+
Hg Bromophenol Blue	Proteins	Blue	+ *	± *	+ *	+ *	+ *	+ *

513 *Results: (-) absent; (±) scarce, (+) intense, and (++) very intense;*

514 **positive response for the cytoplasm of the secreting cells*

515

Figure 1


a
b
1 mm
c
200 µm
d
e
f
25 µm
g
25 µm
h
25 µm
SER
p
p
0.5 µm
i
cu
pc
SER
0.5 µm
j
RER
D
V
1 µm
k