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Growing basil in the underwater biospheres of Nemo's Garden®: Phytochemical, physiological and micromorphological analyses

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ABSTRACT

The need for alternative cultivation methods is urgent for regions of the world where cultivable land is scarce: underwater areas are unexploited and vast. Nemo's Garden® Project aims at creating a green and alternative agriculture system: its biospheres are underwater greenhouses, developed for areas where plants growth is difficult in terrestrial conditions, due to climate change. Basil was chosen as model plant to study its phytochemical, physiological, and micromorphological parameters, in comparison with the same plants grown in terrestrial conditions in the Sanremo Research Centre for Vegetable and Ornamental Crops (CREA-Centro di Ricerca Orticoltura e Florovivaismo) greenhouse. While the micromorphological analyses showed no detectable differences between control and biospheres samples, the phytochemical investigations evidenced a switch of the essential oil chemotype from methyl eugenol/linalool to methyl eugenol, respectively. The headspaces 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: total chlorophyll, total carotenoids and total polyphenols were present in higher amounts in the biospheres samples, with a 31.52% and 13.3% increase in the antioxidant activity and polyphenols content, respectively. Basil was well adapted in the biosphere environment, whose influence should be studied in different species to assess the viability of an industrial scale-up of the Nemo's Garden®.

1. Introduction

The Intergovernmental Panel on Climate Change (IPCC) has already entered its Sixth Assessment Cycle and its 2014 Climate Change Synthesis Report stated that "*scientific evidence for warming of the climate system is unequivocal (…) The atmosphere and ocean have warmed, the amounts of snow and ice have diminished, and sea level has risen*" (IPCC, 2014). The changes in temperature, precipitation, and carbon dioxide, as well as climate variability and surface water draining are already challenging for agriculture, and they are expected to affect it even more in the future, both in the Southern and Northern countries (Juhola et al., 2017; Karimi et al., 2018).

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 represent 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 et al., 2016). They are air-filled domes made of acrylic (transparent plastic material), holding approximately 2000 L of air, anchored to the bottom of the sea by many chains, floating from 5 to 10 m depth in front of the shoreline of the Noli town, close to Savona, Italy (Fig. 1).

Nemo's Garden® Project started in 2012, but the systematic study on the influence of the marine environment on plants grown underwater began in 2015. Several plant species were cultivated in the Nemo's

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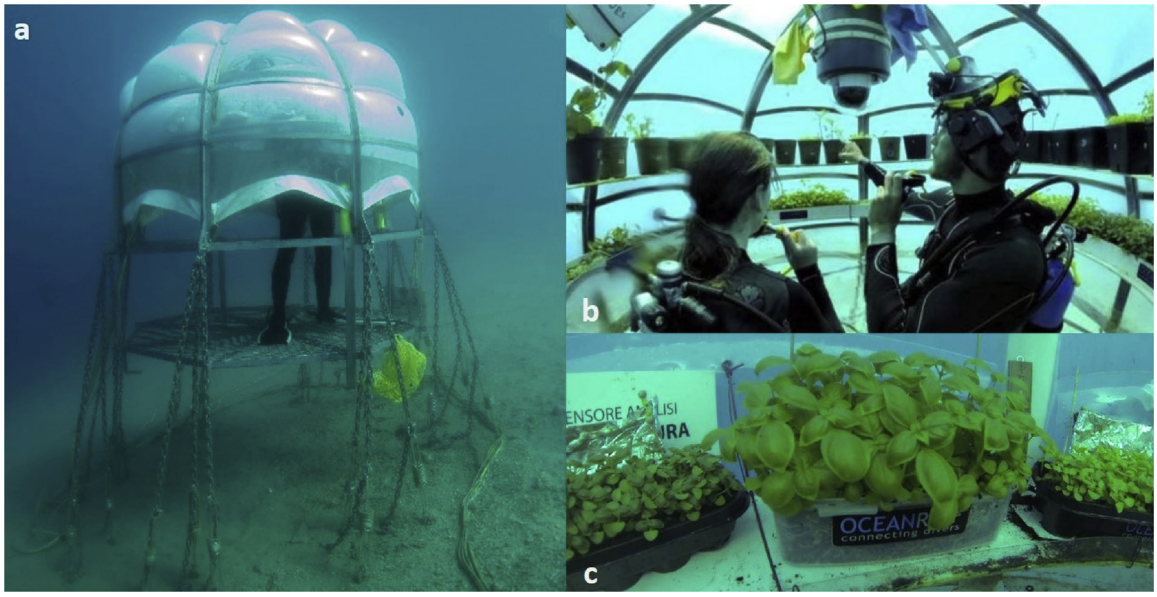

Fig. 1. The Nemo's Garden® underwater farm. a) The biosphere anchored to the bottom of the sea. b) Inside the air-filled biosphere. c) One of the basil specimens analysed in the present study.

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 Research Centre for Vegetable and Ornamental Crops (CREA-Centro di Ricerca Orticoltura e Florovivaismo) 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: basil was chosen as a model specimen, due to its importance for the farmers and in the Ligurian cuisine.

Basil (*Ocimum basilicum* L.) is an annual culinary herb of the Lamiaceae family. Whilst it is native to Asia, it is largely grown in Italy, where it is extensively used in the typical Italian cuisine: it is the main ingredient of the Ligurian Pesto sauce. The trade importance of basil 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 et al., 2005; Politeo et al., 2007). The leaves and flowering tips or apex of the plant are reported as carminative, galactogogue, stomach-aiding and anti-spasmodic in folk medicine and its essential oil showed antimicrobial and antioxidant activities (Hussain et al., 2008). Moreover, basil contains phenolic antioxidant compounds, free radical-scavengers, and metal chelators (Sgherri et al., 2010). Beyond culinary consumption, aromatic herbs such as basil are important sources of value-added products like essential oils (EOs), whit several industrial purposes (i.e. pharmaceutical, cosmetic, pest management, etc.).

The FAO climate biome classification (http://ecocrop.fao.org) reports basil as a species that 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 with a characteristic aroma and taste determined by a pool of several compounds (Lee et al., 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). Moreover, 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).

2. Materials and methods

2.1. Plant material and growth conditions

In the underwater farm, seeding occurred on 25 August 2017. 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 2017 and brought to the surface with the aid of pressurized cases: their collection has been performed at such an early (9 weeks of age) stage of growth of the plants because the Ligurian pesto sauce recipe requires young basil leaves. 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 lx (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.

The control samples were sown at CREA in Sanremo on 19 August 2017 in 1-L plastic pots filled with mineral wool and coconut fiber 50:50 v/v. The plants were grown in a greenhouse until 15 October 2017, when the samples were collected: their collection has been performed at the same phenological stage of the biosphere samples. 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 23,000 and 35000 lx (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.

2.2. 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 h. The hydrodistillations have been performed on 80 g of fresh leaves taken from different specimens to obtain statistically significant samples.

Immediately after the extraction, 1 μL of essential oil was injected after 5% dilution in *n*-hexane HPLC grade for each replication.

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 for sampling the headspace of the samples. 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. Four fresh leaves (without stalks) for each sample were inserted in a glass vial, which was then closed with aluminum foil. After 30 min of equilibration time, the fiber was exposed to the headspace for 2 min at room temperature. 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. Triplicates were performed for each analysis.

2.2.3. Gas chromatography – mass spectrometry analyses and peaks identification

The GC/EI-MS analyses were performed with a Varian CP-3800 apparatus equipped with a DB-5 capillary column (30 m X0.25 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 acquisition parameters were as follows: full scan; scan range: 35–300 m/z; scan time: 1.0 s; threshold: 1 count.

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 (Adams, 1995; Adams et al., 1997; Davies, 1990; Jennings and Shibamoto, 1982; Masada, 1976; Swigar and Silverstein, 1981).

2.2.4. Statistical analyses

The percentage of dissimilarity contribution of all the compounds in essential oil and in the headspaces was evaluated by means of the Similarity Percentage test (SIMPER) with the Bray-Curtis distance/similarity measure. The statistical significance of the difference in the relative abundances of the compounds accounting for at least 1.00% in the dissimilarity rate of the emissions was evaluated using the F- or T-test, for compounds with equal or unequal variances, respectively. The SIMPER, F- and T-tests were performed with the Past 3.20 Software (Hammer et al., 2001).

The analysis of variance (ANOVA) analyses were carried out using the JMP software package (SAS Institute, Cary, NC, USA). The complete composition replicates of both the essential oils and the headspaces were transformed using arcsine square root (arcsin √x) for normalization and then subjected to analysis of variance (ANOVA) to obtain mean values and confidence intervals ($\alpha = 0.05$). Averages were separated by Tukey's b post hoc test. $P < 0.05$ was used for the significance of differences between means.

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.

2.3.2. Total phenolic compounds

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

2.3.3. DPPH scavenging ability

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

$$\% \text{ inhibition} = (A_{blank}\text{-}A_{sample} / A_{blank}) \text{ x100w}$$

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

2.4. Micromorphological analyses

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

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

2.4.1. SEM investigation

Plant material was first hand-prepared, fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 5 days, dehydrated in an ascending ethanol series up to absolute and then dried using a critical-point-dryer apparatus. The samples, mounted on aluminum stubs, were coated with gold and observed with a Philips XL 20 SEM operating at 10 kV.

2.4.2. LM investigation

The samples were frozen, sectioned and stained with various histochemical techniques to evidence the chemical nature of the secretory products and to specifically locate the sites of terpene accumulation and release. The following methods were employed: Fluoral Yellow 088 for total lipids (Brundrett et al., 1991), Nile Red for neutral lipids

(Greenspan et al., 1985), Nadi reagent for terpenes (David and Carde, 1964), Ruthenium Red (Jensen, 1962) and Alcian Blue (Beccari and Mazzi, 1966) for acidic polysaccharides, Mercuric Bromophenol Blue for proteins (Mazia et al., 1953), Ferric Trichloride for polyphenols (Gahan, 1984) and Aluminium Trichloride for flavonoids (Mazia et al., 1953). Control procedures were carried out at the same time. Observations were made with a Leitz DM-RB Fluo optical microscope.

2.4.3. TEM investigation

Small segments of plant material were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 and post-fixed in 2% OsO_4 for 1 h, dehydrated in ethanol in ascending grades up to absolute, and embedded in Spurr's resin. Ultrathin sections were stained with uranile acetate and lead citrate. Samples were examined with a Philips EM-300 TEM.

3. Results and discussion

3.1. Phytochemical investigation

3.1.1. Essential oil compositions

The essential oil (EO) extraction yield of the control basil sample was 0.016% w/w. Its complete composition is reported in Table 1, where 42 compounds have been identified in total. The detected chemotype was methyl eugenol/linalool: the former is a phenylpropanoid, which accounted for 21.99% (Table 1), whilst the latter is an oxygenated monoterpene with a relative abundance of 19.35%. These two classes of compounds were the most relevant ones: combined, they represented more than 60% of the total composition, as each one accounted for more than 30%. Eugenol, another phenylpropanoid, followed with a relative abundance of 8.38%. Among oxygenated monoterpenes, 1,8-cineole showed a relevant presence (6.71%). Sesquiterpene hydrocarbons were the third most represented chemical class (27.08%), of which *trans*-α-bergamotene was the most abundant one (7.31%). The statistical analysis (SIMPER test, Table 2) evidenced that 15 compounds contributed more than 1% to the dissimilarity between the two samples. All of these compounds showed a statistically significant ($P < 0.05$) difference in their relative abundances between the control and the Nemo's Garden sample. Furthermore, over 55% of the total dissimilarity among the two EOs was due to only three compounds (methyl eugenol, linalool and eugenol).

Basil shows a wide number of chemotypes, with cultivars and geographical origin being the main reasons for such a variability in its essential oil composition. An overview of some of the numerous published EO-based basil chemotype classifications is shown in Table 3. Özcan and Chalchat (2002) reported the composition of a Turkish *Ocimum basilicum* mainly rich in methyl eugenol, accounting for 78.02% of the essential oil. The relevant presence of methyl eugenol in basil essential oil has been studied by Miele et al. (2001), who reported a negative correlation on plants height and methyl eugenol relative abundance, since in young specimens (up to 10–12 cm) methyl eugenol showed a more relevant presence than in older (and taller) ones. In the present study, both control and Nemo's Garden® samples were young specimens, approximately 7 cm high: as well as a chemotype matter, this could be the reason of such a relevant methyl eugenol relative abundance.

The composition of Nemo's Garden® basil essential oil (extraction yield 0.025% w/w), in which 33 compounds have been detected, showed a methyl eugenol chemotype, with this phenylpropanoid representing 49.6% of the essential oil (Table 1). The very same species of basil grown in different environmental conditions showed a shift of chemotype: from methyl eugenol – linalool to methyl eugenol, as linalool only accounted for 1.3%. In comparison with the control sample, eugenol relative abundance was more than doubled. Moreover, differently from the control sample, sesquiterpene hydrocarbons were the second most abundant (19.1%) chemical class of compounds, with α-humulene (5.9%) and *trans*-α-bergamotene (4.7%) showing the largest abundance.

Table 1
Compositions of the essential oils hydrodistilled from the control sample and the Nemo's Garden basil.

Constituents	l.r.i.[1]	Relative abundance (%) ± SD	
		Nemo's Garden	Control
(*E*)-2-hexenal	856	-[2]	0.14 ± 0.19
α-pinene	941	0.11 ± 0.00	0.21 ± 0.01
sabinene	976	0.15 ± 0.00	0.34 ± 0.01
β-pinene	982	0.32 ± 0.00	0.72 ± 0.04
myrcene	993	0.18 ± 0.01	0.83 ± 0.01
octanal	1001	–	0.11 ± 0.01
δ-3-carene	1011	–	0.13 ± 0.01
1,8-cineole	1034	4.87 ± 0.04	6.71 ± 0.27
(*E*)-β-ocimene	1052	0.63 ± 0.00	1.22 ± 0.06
cis-sabinene hydrate	1070	0.17 ± 0.01	0.27 ± 0.01
terpinolene	1088	0.30 ± 0.01	0.76 ± 0.04
linalool	1101	1.28 ± 0.01	19.35 ± 1.03
1-octen-3-yl acetate	1111	–	0.21 ± 0.01
camphor	1143	0.65 ± 0.00	0.61 ± 0.04
δ-terpineol	1170	0.15 ± 0.01	0.14 ± 0.01
α-terpineol	1191	0.64 ± 0.01	0.80 ± 0.01
methyl chavicol	1197	0.78 ± 0.01	–
n-octanol acetate	1214	0.24 ± 0.01	0.44 ± 0.01
*iso*bornyl acetate	1285	0.96 ± 0.01	2.70 ± 0.08
α-cubebene	1351	–	0.31 ± 0.43
α-terpinyl acetate	1352	–	0.18 ± 0.01
eugenol	1358	17.14 ± 0.04	8.38 ± 0.40
α-copaene	1376	–	0.16 ± 0.01
(*E*)-methyl cinnamate	1380	0.74 ± 0.01	–
β-cubebene	1390	–	0.31 ± 0.43
β-elemene	1392	0.19 ± 0.01	0.20 ± 0.04
methyl eugenol	1403	49.54 ± 0.08	21.99 ± 1.12
β-caryophyllene	1420	0.87 ± 0.01	0.19 ± 0.01
trans-α-bergamotene	1438	4.73 ± 0.01	7.31 ± 0.15
cis-muurola-3,5-diene	1447	–	0.07 ± 0.10
α-humulene	1456	5.90 ± 0.01	1.68 ± 0.12
(*E*)-β-farnesene	1460	3.08 ± 0.01	5.80 ± 0.89
cis-muurola-4(14),5-diene	1462	–	0.06 ± 0.08
γ-muurolene	1477	1.97 ± 0.01	–
germacrene D	1478	–	2.78 ± 0.16
(*E*,*Z*)-α-farnesene	1490	–	1.12 ± 0.58
bicyclogermacrene	1495	0.49 ± 0.04	2.13 ± 0.35
α-bulnesene	1505	1.35 ± 0.03	2.69 ± 0.54
trans-γ-cadinene	1513	0.24 ± 0.01	1.54 ± 0.34
β-sesquiphellandrene	1524	0.25 ± 0.00	0.78 ± 0.42
(*E*)-γ-bisabolene	1535	0.12 ± 0.01	–
(*Z*)-3-hexenyl benzoate	1570	–	0.09 ± 0.12
caryophyllene oxide	1581	–	0.28 ± 0.08
viridiflorol	1590	–	0.10 ± 0.13
1,10-*di*-*epi*-cubenol	1614	–	0.57 ± 0.04
epi-α-cadinol	1640	0.55 ± 0.01	5.44 ± 0.72
β-eudesmol	1650	0.46 ± 0.01	–
methyl-*p*-methoxycinnamate	1692	0.13 ± 0.01	–
(*E*,*E*)-farnesyl acetate	1843	0.59 ± 0.02	–
Monoterpene hydrocarbons		1.68 ± 0.01	4.18 ± 0.08
Oxygenated monoterpenes		8.70 ± 0.06	30.74 ± 1.44
Sesquiterpene hydrocarbons		19.16 ± 0.06	27.08 ± 3.29
Oxygenated sesquiterpenes		1.59 ± 0.04	6.38 ± 0.54
Phenylpropanoids		68.33 ± 0.11	30.37 ± 1.52
Other non-terpene derivatives		0.36 ± 0.18	1.03 ± 0.36
Total identified (%)		99.8 ± 0.24	99.76 ± 0.07

[1] Linear retention indices on a DB5 column; [2]Not detected.

The shading conditions in the biospheres are heavy, as the light intensity that reaches the plants is reduced by 80–90% (152–190 μmol/m^2s) in comparison with unshaded greenhouse conditions (600–1600 μmol/m^2s on average (Chang et al., 2008)) due to the water depth and the biosphere material. Chang et al. (2008) studied the behavior of the three major compounds (eugenol, methyl eugenol and linalool) in the essential oils hydrodistilled from *O. basilicum* cv. 'Basil Sweet Genovese' grown under different shading conditions obtained

Table 2
Similarity percentages (SIMPER) test for the compositions of the EOs extracted from the control and the Nemo's Garden basil samples.

Compounds	Average dissimilarity	Individual contribution %	Cumulative contribution %
methyl eugenol	13.81	29.9	29.9
linalool	9.059	19.61	49.51
eugenol	4.393	9.51	59.02
epi-α-cadinol	2.453	5.311	64.33
α-humulene	2.116	4.58	68.91
germacrene D	1.392	3.013	71.92
(*E*)-β-farnesene	1.365	2.956	74.88
trans-α-bergamotene	1.292	2.796	77.68
γ-muurolene	0.9876	2.138	79.81
1,8-cineole	0.9241	2	81.81
*iso*bornyl acetate	0.874	1.892	83.71
bicyclogermacrene	0.8222	1.78	85.49
α-bulnesene	0.6718	1.454	86.94
trans-γ-cadinene	0.6517	1.411	88.35
(*E*,*Z*)-α-farnesene	0.5615	1.215	89.57

Table 3
Overview of some published reports on essential oil-based basil chemotypes classifications.

Source	EO Chemotypes
(Lawrence, 1988)	1 Methyl chavicol
	2 Linalool
	3 Methyl eugenol
	4 Methyl cinnamate
(Grayer et al., 1996)	1 Linalool
	2 Methyl chavicol
	3 Linalool/methyl chavicol
	4 Linalool/eugenol
	5 Methyl chavicol/methyl eugenol
(De Masi et al., 2006)	1 Linalool > methyl chavicol > eugenol
	2 Methyl chavicol > linalool > eugenol
	3 Linalool > eugenol > methyl chavicol
	4 Linalool > methyl cinnamate > methyl chavicol > eugenol
	5 Citral (neral + geranial) > linalool
(Zheljazkov et al., 2008)	1 Linalool
	2 Linalool/eugenol
	3 Methyl chavicol
	4 Methyl chavicol/linalool
	5 Methyl eugenol/linalool
	6 Methyl cinnamate/linalool
	7 Bergamotene
(Koutsos et al., 2009)	1 European: linalool/methyl chavicol
	2 Reunion: methyl chavicol
	3 Tropical: methyl cinnamate
	4 Java: eugenol
(Carović-Stanko et al., 2011)	1 Linalool
	2 Linalool/eugenol
	3 Linalool/(*Z*)-methyl cinnamate
	4 Methyl chavicol/linalool
	5 Methyl chavicol
(Liber et al., 2011)	1 Linalool/eugenol
	2 Linalool
	3 Methyl chavicol/linalool
	4 (*Z*)-Methyl cinnamate
	5 Methyl chavicol

with shading nets. High daily light integrals significantly increased linalool and eugenol relative abundances, whilst methyl eugenol showed a relevant increment with lower daily light integrals. The light intensity, instead, did not influence other aroma active compounds of basil, like 1,8-cineole. In accordance with the latter, in the present study, linalool showed a significant decrement from the control (19.35%) to the Nemo's Garden® (1.38%) sample and methyl eugenol evidenced a more than two-fold enhancement (from 21.99% in the control conditions to 49.54% in the Nemo's biospheres). However, in the studied basil, eugenol showed an increment from control (8.38%) to underwater biosphere (17.14%) conditions. This increase could be due to the differences in the red/far-red ratio, as the light quality is as important a parameter as the light intensity (Morelli and Ruberti, 2002). The total amount of essential oil significantly increased with the increment of the radiant energy, particularly detected in the case of the most important flavor compounds 1,8-cineole, linalool, and eugenol. Moreover, the level of the main compound, methyl eugenol, decreased. This result is important because this compound is of toxicological concern to human health, due to its structural similarity to known carcinogenic phenylpropanoids, such as methyl chavicol (Nitz and Schnitzler, 2004).

Table 4
Complete compositions of the headspaces of the Nemo's Garden basil compared to the control sample.

Constituents	l.r.i.[1]	Relative abundance (%) ± SD	
		Nemo's Garden	Control
α-pinene	941	-[2]	1.39 ± 0.40
camphene	954	–	0.32 ± 0.45
β-pinene	982	3.65 ± 0.02	6.83 ± 0.05
myrcene	993	–	4.18 ± 0.19
p-mentha-1(7),8-diene	1004	–	0.13 ± 0.18
δ-3-carene	1011	–	0.90 ± 0.00
1,8-cineole	1034	25.52 ± 0.11	42.96 ± 0.04
(*E*)-β-ocimene	1052	0.86 ± 0.01	6.46 ± 0.01
cis-sabinene hydrate	1070	0.35 ± 0.00	0.13 ± 0.00
terpinolene	1088	1.57 ± 0.01	2.95 ± 0.01
linalool	1101	0.29 ± 0.01	7.15 ± 0.01
camphor	1143	0.68 ± 0.01	0.77 ± 0.00
α-terpineol	1191	0.48 ± 0.01	0.42 ± 0.01
δ-elemene	1340	0.55 ± 0.00	0.20 ± 0.01
α-cubebene	1351	–	0.05 ± 0.07
eugenol	1358	1.93 ± 0.01	0.85 ± 0.01
α-copaene	1376	0.37 ± 0.00	0.22 ± 0.01
β-cubebene	1390	–	0.15 ± 0.01
β-elemene	1392	3.88 ± 0.01	1.02 ± 0.01
methyl eugenol	1403	2.55 ± 0.01	3.83 ± 0.01
cis-α-bergamotene	1416	0.41 ± 0.00	–
β-caryophyllene	1420	2.26 ± 0.01	0.46 ± 0.00
trans-α-bergamotene	1438	26.15 ± 0.11	7.25 ± 0.01
aromadendrene	1441	0.17 ± 0.00	–
α-humulene	1456	17.39 ± 0.08	0.87 ± 0.00
(*E*)-β-farnesene	1460	–	6.01 ± 0.01
cis-muurola-4(14),5-diene	1462	0.33 ± 0.00	–
γ-muurolene	1477	4.55 ± 0.01	–
germacrene D	1478	–	1.29 ± 0.01
(*E*,*Z*)-α-farnesene	1490	–	0.41 ± 0.01
bicyclogermacrene	1495	1.38 ± 0.01	0.64 ± 0.01
α-bulnesene	1505	2.41 ± 0.01	0.60 ± 0.01
β-bisabolene	1509	–	0.12 ± 0.00
trans-γ-cadinene	1513	1.05 ± 0.01	0.63 ± 0.01
β-sesquiphellandrene	1524	0.29 ± 0.41	0.25 ± 0.02
epi-α-cadinol	1640	0.17 ± 0.24	–
Monoterpene hydrocarbons		6.07 ± 0.04	23.69 ± 0.13
Oxygenated monoterpenes		27.31 ± 0.13	51.43 ± 0.04
Sesquiterpene hydrocarbons		61.18 ± 0.14	20.14 ± 0.06
Oxygenated sesquiterpenes		0.17 ± 0.24	–
Phenylpropanoids		4.48 ± 0.03	4.67 ± 0.01
Total identified (%)		99.21 ± 0.30	99.92 ± 0.12

[1] Linear retention indices on a DB5 column; [2]Not detected.

3.1.2. Headspace solid phase micro-extraction (HS-SPME)

The spontaneous volatile emission of the aerial parts of the control sample, in which 31 compounds have been identified, was mainly rich in monoterpenes, which cumulatively reach 75.12% of the total headspace. The oxygenated ones represented half of the total emission (51.43%, Table 4): 1,8-cineole was the most abundant volatile compound, accounting for 42.96%, followed by linalool (7.15%). The most quantitatively relevant monoterpene hydrocarbons were β-pinene (6.83%) and (*E*)-β-ocimene (6.46%). Among sesquiterpene

hydrocarbons, *trans*-α-bergamotene was the most abundant (7.25%).

In the headspace of Nemo's Garden® basil, the aroma profile was composed of 25 compounds, of which sesquiterpene hydrocarbons were the most abundant chemical class, reaching up to 61.18%. Among these, *trans*-α-bergamotene was the most relevant (26.15%), followed by α-humulene (17.39%). Oxygenated monoterpenes were significantly represented (27.31%) in this sample headspace, as well, and 1,8-cineole alone represented most of this class relative abundance, as it accounted for 25.52%. Phenylpropanoids showed a similar relevance in both samples: 4.67 and 4.48% in the control and Nemo's Garden® basil, respectively.

(*E*)-β-Farnesene was significantly represented in the control sample, where it accounted for up to 6.01%, whilst it was not detected in the biosphere sample. The opposite behavior was shown by γ-muurolene: it was not detected in the control sample, while it reached 4.55% in the Nemo's basil headspace.

These divergent emission profiles are due to the different growth environment conditions. Besides the metabolic changes induced by the different light and humidity, the biospheres represent a closed and protected environment, in which no pollinators, nor parasites, are present. These environmental conditions influence the volatile emission, which is the fastest plant response to its habitat.

The similarity percentages (SIMPER) test (Table 5) evidenced 17 compounds individually contributing at least 1% to the total dissimilarity between the two headspaces, with only four compounds (*trans*-α-bergamotene, 1,8-cineole, α-humulene and linalool) contributing over 55% of the total dissimilarity. All of these 17 compounds showed a statistically significant ($P < 0.05$) difference in their relative abundances between the control and the Nemo's Garden sample.

3.2. Physiological investigation: metabolites analyses

The analysis of photosynthetic pigments (total chlorophyll and total carotenoids) showed that basil plants grown in Nemo's Garden® have higher amounts of these compounds than control plants (Table 6). This can be due to the lower level of irradiance of Nemo's plants, so that the photosynthetic pigments were more concentrated to counteract the low efficiency of the light. This reflects the examination of the Chla/Chlb ratio. In control plants, the ratio was quite normal (2.63), and the content of Chlorophyll a (Chla), the most important for the photons capture, was in good balance with the amount of Chlorophyll b (Chlb). On the other hand, Nemo's plants showed a lower Chla/Chlb ratio (1.1), although the higher concentration of pigments. This ratio is common for plants living in shaded condition, where the ratio is around 1.3 due to the increase of Chlb concentration (Liu et al., 2004; Ruban, 2015). The Chlb is located in the light-harvesting complexes of PS II and PS 1, and Chla is a component of this complex as well as of PS I and PS II core complexes, so in low efficiency of light plants increased the Chlb to enhance the photon capture (Ruban, 2015).

Table 5
Similarity percentages (SIMPER) test for the compositions of the headspaces of the control and the Nemo's Garden basil samples.

Compounds	Average dissimilarity	Individual contribution %	Cumulative contribution %
trans-α-bergamotene	9.518	18.62	18.62
1,8-cineole	8.781	17.18	35.8
α-humulene	8.316	16.27	52.07
linalool	3.454	6.758	58.83
(*E*)-β-farnesene	3.024	5.917	64.75
(*E*)-β-ocimene	2.821	5.52	70.27
γ-muurolene	2.291	4.482	74.75
myrcene	2.103	4.115	78.87
β-pinene	1.601	3.133	82
β-elemene	1.44	2.817	84.82
α-bulnesene	0.913	1.786	86.6
β-caryophyllene	0.9063	1.773	88.38
α-pinene	0.6979	1.365	89.74
terpinolene	0.6932	1.356	91.1
germacrene D	0.6478	1.268	92.37
methyl eugenol	0.6428	1.258	93.62
eugenol	0.5455	1.067	94.69

Table 6
Determination of foliar pigments (chlorophyll a, chlorophyll b, total chlorophylls) and total carotenoids (mg/g FW), total polyphenol content (mg/g DW GA equivalent), IC50 of the free radical (DPPH) scavenging activity of 9 weeks-old basil plants collected in Nemo's Garden® and in control plants grown in terrestrial aerial condition. Mean values were obtained from three 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	0.037	0.05
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

Carotenoids were affected by the cultivation under water, showing an increase (by 2.5 times) in Nemo's leaves. The carotenoids/chlorophylls ratio may often be a good indicator of stress in plants (Hendry and Price, 1993). In this trial, the ratio was lower, indicating that no photoprotection is needed in the underwater environment and probably reflects the adaptation of plants to new growing condition.

To better analyze the avoidance of some stress conditions, total polyphenols have been determined. Total polyphenols content was 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) was 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 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 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. 2a-e).

The non-glandular hairs were short, simple, uniseriate, with a pointed apex and a smooth cuticular surface; they were predominantly located on the median and secondary ribs of the abaxial leaf surface (Fig. 2a-c). As regards to the glandular trichomes, peltates and two basic types of capitates have been observed (Fig. 2).

The peltates were constituted by one or two basal epidermal cells, one neck cell and by a four-celled secreting head (40–60 μm in diameter, Fig. 2d-e), surmounted by a wide subcuticular space where the

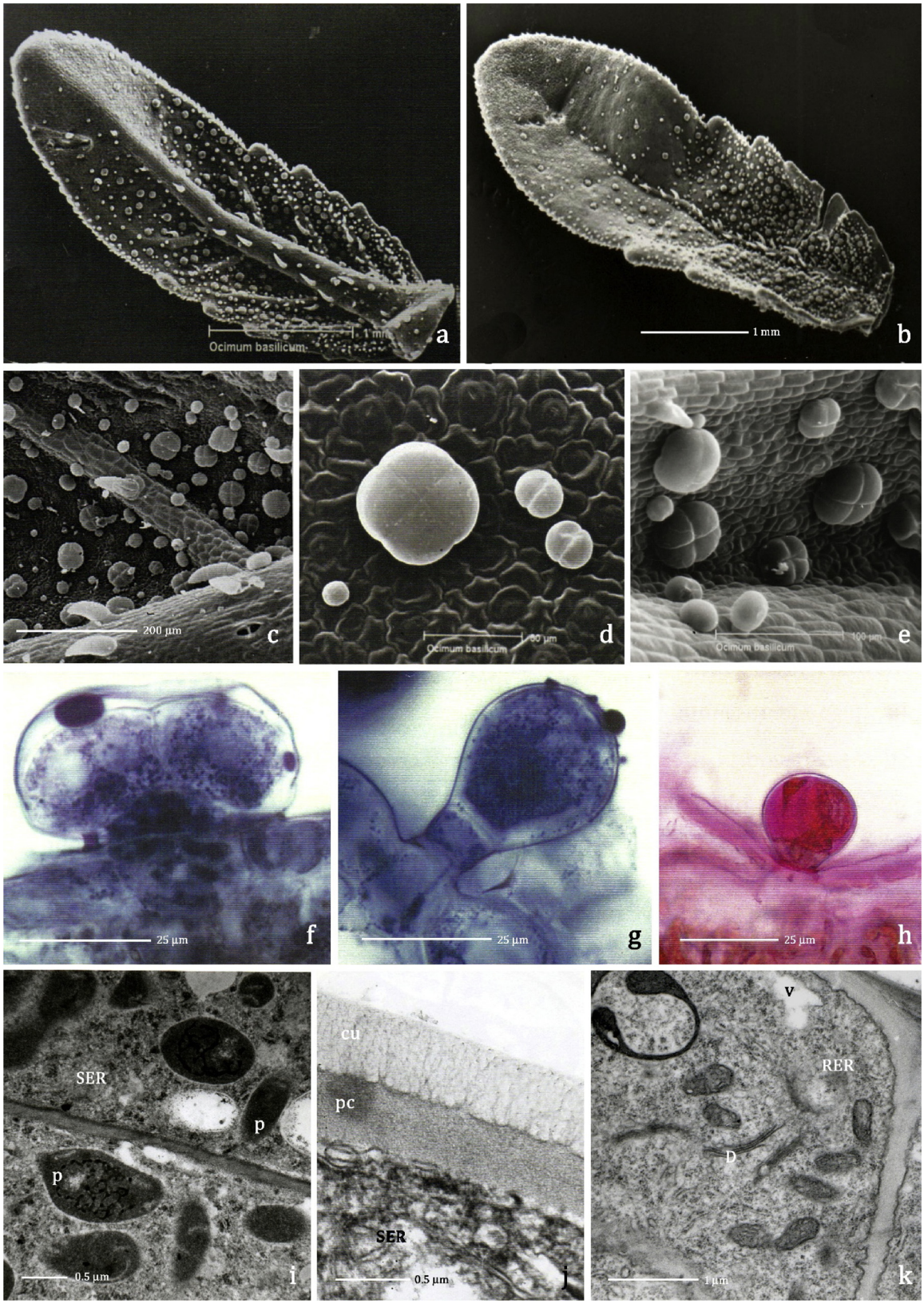

Fig. 2. a–c. Trichome distribution pattern in the Nemo's samples of *Ocimum basilicum*, SEM. a, b. Overviews of the leaf abaxial (a) and adaxial (b) surfaces. c. Particular of the leaf abaxial surface. d–e. Trichome morphotypes in *Ocimum basilicum* (peltates, type I and II capitates) in the Nemo's sample (d) and in the control (e). f–h. Histochemistry of the glandular trichomes in *Ocimum basilicum*, LM: f–g. Nadi reagent in the peltate (f) and in the type I capitate (g); h. PAS reaction in the type II capitate. 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 (j); secreting cell cytoplasm of a type II capitate (k).
Symbols: cu, cuticular layer; D, dictyosomes; p, plastid; pc, pectic-celluosic layer; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; v, vacuole.

Table 7
Histochemical results on the leaf glandular trichomes of the control and Nemo's plants of *Ocimum basilicum* L.

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	+ *	± *	+ *	+ *	+ *	+ *

Results: (-) absent; (±) scarce, (+) intense, and (+ +) very intense; *positive response for the cytoplasm of the secreting cells.

secreted material accumulates; the breakage of the outer cuticle was occasionally observed (Fig. 2c). The capitate trichomes were formed by a basal epidermal cell, one neck cell and by one or two apical secreting cells. The diameter of the glandular head was about 20–25 µm, while the trichome length was approximately 30 µm (Fig. 2d-e). The diversity in head morphology allowed the recognition of two types of capitate hairs: type I with a unicellular head and type II with a bicellular head (Fig. 2d-e), the first being sporadic.

The leaf primordia showed a high density of glandular trichomes at the proximal and middle regions, while the distal portion appeared mostly hairless. With the ongoing of leaf development, trichomes density decreased. The adaxial and abaxial surfaces exhibited a homogeneous distribution pattern (Fig. 2a-b): capitates were preferably located along the veinal system, whereas peltates were uniformly distributed over the entire lamina.

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

3.3.2. LM investigation

The results of the histochemical investigation are reported in Table 7. The chemical nature of the secretory products of all the glandular trichomes proved uniform in the control and Nemo's plants.

The peltates exhibited great affinity for the dyes specific for lipophilic substances. Indeed, intense orange and yellow-greenish colorations of the secretory products resulted following the application of Nile Red and Fluoral Yellow-088, respectively. The NADI reagent, specific for terpenes, displayed a strong positive response (Fig. 2f). The dyes for total phenols and flavonoids evidenced the cytoplasm of the secreting cells.

Type I capitate trichomes showed an exclusive positive response to the NADI reagent, which highlights the glandular head and few droplets of secreted material outside the apical periclinal wall (Fig. 2g). The secreted material of type II capitates showed affinity only for the dyes specific for polysaccharides (PAS reaction, Fig. 2h) and proteins.

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

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

3.3.3. TEM investigation

TEM observations involved the secreting cells of mature peltate and of type II capitate trichomes (Fig. 2i-k): they confirmed the preliminary histochemical results.

In all the types of glandular hairs, numerous plasmodesmata crossed the periclinal walls between all the cells constituting the trichome and the anticlinal walls of the secreting head. This ultrastructural feature evidenced that all the trichome cells are involved in the production and release of the secreted material.

In the active peltate trichomes, the most striking ultrastructural feature was the occurrence of numerous plastids with an irregular internal membrane system and evident plastoglobuli associated to periplastidial smooth endoplasmic reticulum (Fig. 2i). In the area below the subcuticular space the plasmalemma was crenulated and slightly detached from the wall, forming a thin periplasmatic space in which small vesicles are visible (Fig. 2j). At this stage, the well-developed subcuticular space contained materials of different appearance: small electrondense globules of lipophilic nature, immersed in an abundant granular matrix, presumably constituted by phenols. This evidence confirmed the results of the histochemical tests as abundant plastids and smooth endoplasmic reticulum are the cell compartments responsible for the production and transport of terpenic substances, which are among the main components of the essential oil (Hallahan, 2000).

At the active secretory phase, the secreting cell cytoplasm of the type II trichomes was characterized by abundant dictyosomes, originating a large number of vesicles, and by a well-developed rough endoplasmic reticulum often surrounding vacuoles (Fig. 2k). These ultrastructural features and the histochemical results of the PAS reaction indicated the production of polysaccharides (Giuliani and Maleci Bini, 2008). In addition, the occurrence of rough endoplasmic reticulum in association with dictyosomes suggests that the polysaccharidic secretion is associated with the synthesis of proteic material.

4. Conclusion

The most evident phytochemical modifications to the biosphere growth conditions are the essential oil chemotype switch and the very different spontaneous emission patterns, highlighting the fast response of the plant to the new habitat. The differences in the spontaneously emitted volatiles were more apparent than in the essential oils when compared to 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. Therefore, it may be stated that the analyzed 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 Nemo's Garden® underwater farm represents a promising alternative system to standard agriculture meant for areas where the cultivable soil is scarce or the climatic conditions are not suitable for some species. Indeed, the underwater farm provides a new environment for plants to grow. Waterlogging of soils is crucial in certain areas of the world, where the rainy season is prolonged or extreme precipitations are concentrated, with the development of flooding conditions. These events determine loss of plant and food harvest and such novel

cultivation technology may contribute to sustain the production of food or herbal products. Further studies are needed to assess the adaptation of distinct species to the marine or lake underwater conditions, especially pressure. This pioneering plant growth system could be applied to grow food and/or spice plants, as well as 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.

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