The Essential Oil of *Coleonema album* (Rutaceae) and of a Photomixotrophic Cell Culture Derived thereof

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The volatile shoot oil of *Coleonema album* was found to be composed of monoterpenes, sesquiterpenes and phenylpropanoids. Heterotrophic and photomixotrophic callus cultures were established from green parts of the plant. A photomixotrophic cell line contained a heterogeneous population of plastids and synthesized an essential oil, which differed qualitatively and quantitatively from the green parts of the intact plant. Substitution of 2,4-D and pCPA by a lower amount of NAA and high kinetin concentrations favoured the formation of essential oil. The heterotrophic callus, when grown on the same nutrient medium, was devoid of any differentiated plastids and of essential oil constituents. Thus, a close correlation of light-induced plastid differentiation and essential oil synthesis was concluded.

Introduction

The biotechnological production of essential oil components used in the food industry, in perfumery and in pharmaceuticals is attractive from a commercial viewpoint [1]. In recent years, tissue cultures of a variety of herbs and of other flavour sources have been established and the production of flavour compounds has been investigated. The number of volatiles identified in plant cell cultures is increasing (review in [2]). Nevertheless, reports on the accumulation of significant amounts of essential oil components are rarely found [3–5], indicating that there are still principal obstacles to produce these secondary metabolites *de novo* by *in vitro* systems at present.

Most higher plants excrete mono- and sesquiterpenes into specialized storage sites, such as glandular hairs and resin ducts. The lack of these products in many cell cultures has been related to the low degree of cellular specialization of the culture. In tissue cultures that do not contain appropriate storage sites, essential oil components may severely disturb membrane functions [6]. In cultures of *Lavandula angustifolia* and *Rosmarinus officinalis* grown under natural daylight, *e.g.,* monoterpenes were detected after regeneration of shoots only [7], indicating that the corresponding genetic information was suppressed in the callus stage.

The present paper describes: (1) the investigation of the volatile constituents of *Coleonema album,* (2) the accumulation of essential oil compounds in a photomixotrophic callus culture initiated from this plant, and (3) the effect of cellular differentiation on secondary product formation.

Experimental

Cell cultures

Coleonema album plants were from the Munich Botanical Garden (greenhouse). Leaves and stems from 7 months old plants were sterilized with 2% w/v NaOCl solution for 10 min. Callus initiation using excised tissue of both organs was tested on 6 different media:

B_5-medium [8], revised MS-medium [9], LS-medium [10], SH-medium [11], MG-medium, originally used for Muscat grape cells [12], and LH-medium: micro- and macronutrients as for LS-medium, vitamins and growth factors as for SH-medium.

Best growth was obtained on SH-medium. After several subcultivations, calli from both stems and leaves showed, as expected, converging characteristics in terms of macroscopic and microscopic appearance and essential oil patterns. In order to clarify the effect of carbon source and growth substances on callus growth and the accumulation

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GLC, gas liquid chromatography; MS, mass spectrometry; NAA, naphthylacetic acid; pCPA *p*-chlorophenoxyacetic acid.

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of secondary products, different amounts of carbohydrates and effectors were added to SH-medium: original SH-medium: 3% sucrose, 0.5 $mg \cdot l^{-1}$ 2,4-D, 2 $mg \cdot l^{-1}$ pCPA, 0.1 $mg \cdot l^{-1}$ kinetin; SH 1: 1% sucrose, 1% lactose, 0.1 $mg \cdot l^{-1}$ NAA, 0.1 $mg \cdot l^{-1}$ kinetin; SH 2: 1% sucrose, 1% lactose, 0.05 $mg \cdot l^{-1}$ NAA, 0.2 $mg \cdot l^{-1}$ kinetin; SH 3: 1% sucrose, 1% lactose, 0.5 $mg \cdot l^{-1}$ NAA, 2 $mg \cdot l^{-1}$ kinetin.

Cells were kept at 25 °C. Photomixotrophic cultures were obtained by illumination using a photoperiod of 16 h white light (6000 lux, Osram HQL/R 80 W). Callus cells were transferred to fresh medium every 5 to 7 weeks, and suspension cells every 4 weeks.

Preparation of the extracts for GLC analysis

Extracts from the green parts of the plant and from callus cultures were prepared immediately after harvesting of the plant material. Callus cells were separated from adhering medium, mixed with the twofold amount per weight of MeOH and 1 ml internal standard (100 µg undecanone $\cdot ml^{-1}$) in a cooled Waring Blendor (30 s). The homogenate was centrifuged (5000 × *g*, 10 min, 4 °C), the supernatant was diluted with a saturated solution of NaCl to a MeOH concentration below 40%. This solution was extracted three times with pentane/diethyl ether (2/1). The dried extract was concentrated at 40 °C using a Vigreux column [13], and submitted to gas chromatography.

Shoots and leaves of the parent plant were cut into MeOH and treated like the callus material.

Determination of chlorophyll

The sum of chlorophyll *a* and *b* was determined spectrophotometrically in an 80% (v/v) acetone extract according to Arnon [14].

Gas liquid chromatography and mass spectrometry

A 25 m × 0.3 mm i.d. fused silica capillary column coated with Carbowax 20 M and a Carlo Erba gas chromatograph Fractovap 4130 were used. Conditions: Temperature program 50 °C 5 min isothermal, 50–190 °C at 2 °C/min, at 190 °C isothermal; carrier gas hydrogen, 1.6 ml/min (70 °C); injector split ratio 1:10.

A 30 m × 0.5 mm i.d. WCOT capillary column coated with Carbowax 20 M and a Carlo Erba gas chromatograph Fractovap 2350 were used for sniffing analysis [15]. Conditions: Temperature program 65 °C 5 min isothermal, 65–190 °C at 2 °C/min; carrier gas hydrogen, 1 ml/min (65 °C); split ratio FID-sniffing port 1:5.

GLC-MS: A Finnigan gas chromatograph 9610, directly coupled with a mass spectrometer (Finnigan 4021, Quadrupol), was used. Spectra were recorded at an electron energy of 70 eV in combination with an Incos data system. Spectra were analyzed either by using reference compounds or by comparison with an internal NBS-library (*ca.* 36,000 spectra); for details see [16].

Electron microscopy

Heterotrophic and photomixotrophic callus cells on SH 3 medium were fixed with 2.5% glutaraldehyde in 0.1 M K-phosphate buffer pH 7.4, 1 h. The tissue was rinsed with phosphate buffer and postfixed with 1% osmium tetroxide/phosphate buffer for 2 h. The material was serially dehydrated in ethanol and embedded in epon. Ultramicrotome sections were stained with lead citrate and viewed with the EM 10 A Zeiss electron microscope.

Results

Cell cultures

As literature contained no data on culturing tissues of *Coleonema album,* different media for callus initiation were examined. Plant sections were explanted in the dark and under illumination. After three weeks callus formation was observed on SH-, MG-, LS-, and LH-medium. No initiation was achieved on B_5- and MS-medium during a period of 12 weeks. Under light conditions green primary calli were obtained. Callus growth and chlorophyll formation were best on SH-medium. All subsequent investigations were, therefore, conducted on this medium. Both the heterotrophic and photomixotrophic callus tissue grew slowly. In the dark, homogeneous smooth grey-coloured tissue was formed; callus developed under light consisted of firm and non-homogeneous aggregates with green, brownish and white cells. By transfer to SH-medium variants with higher cytokinin/auxin ratio this heterogeneity was supported. On SH 3 medium a more friable tissue was formed.

Photomixotrophic callus growing on SH 3 medium had a chlorophyll content of 110 µg/g fresh weight (parent leaves: 315 µg/g fresh weight).

Chemical analysis of volatile compounds

The composition and yield of volatile constituents in *Coleonema album* cell cultures growing on different SH-medium variants were compared with the essential oil of the green parts of the plant. Fig. 1 shows distinct qualitative and quantitative differences between extracts of the tissues from the intact plants and of photomixotrophic callus cultures on media SH 1 and SH 3.

Stems and leaves of the plant. GLC-MS examination of the essential oil of *C. album* led to the detection of 41 constituents, mainly monoterpenes, sesquiterpenes and phenylpropanoids with *Z*-β-ocimene, myrcene, α- and β-pinene, β-phellandrene and the unidentified compound **19** being the major monoterpenes (Table I). According to its molecular weight and fragmentation pattern, component **19** is supposed to be a monoterpene with a keto function. Several compounds of the extract could not be identified by means of GLC-MS, and the complete structural elucidation warrants further investigation. Pinocarveol and verbenone were identified by comparison with literature mass spectral data only. α-Guaiene and germacrene D were the major sesquiterpenes, and eugenol was the main phenylpropanoid compound.

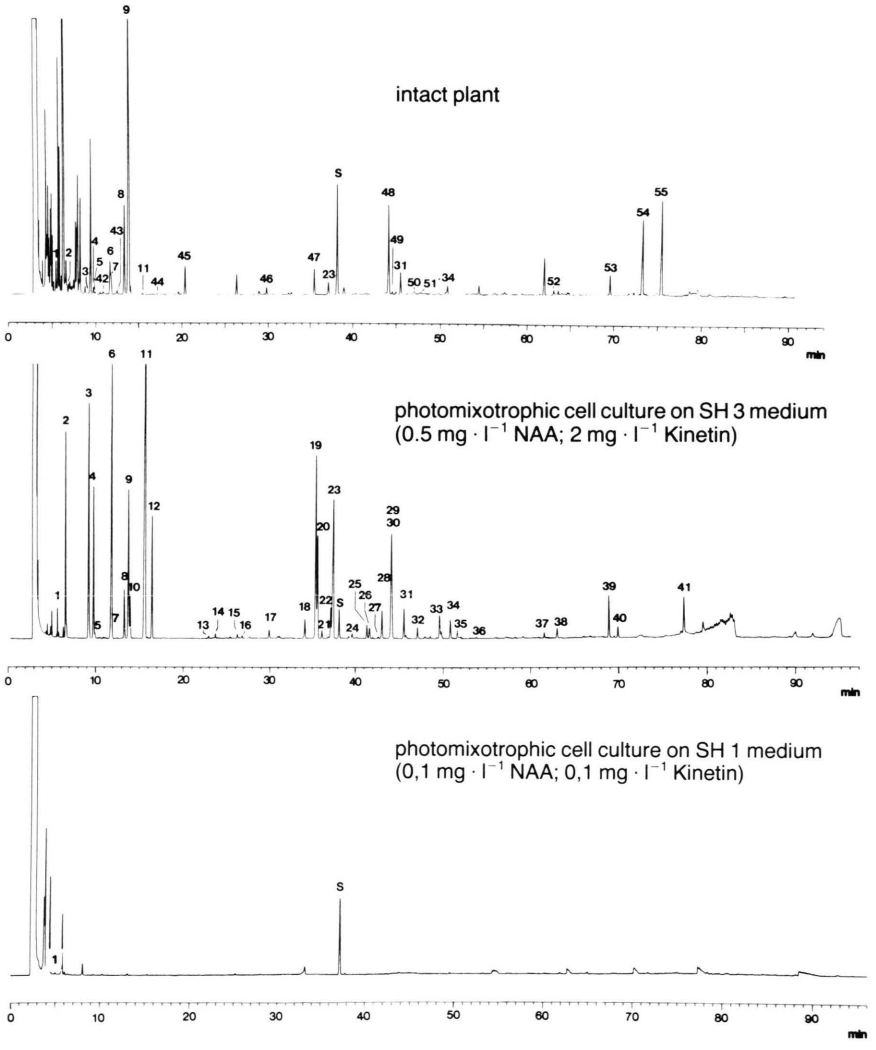

Fig. 1. Capillary GLC-profiles of extracts from the green parts of *Coleonema album* plants and from photomixotrophic calli derived thereof. Peak numbers as in Tables I and II, s = internal standard; chromatographic conditions were as described in "Experimental".

Table I. Compounds identified in the essential oil of green parts of *Coleonema album* plants.

Peak No.	Compound	Quantity [mg/kg fr. w.]	Identification method
1	Ethyl 2-methylbutanoate	15	MS, R
2	α-Pinene	139	MS, R
3	β-Pinene	190	MS, R
4	Sabinene	38	MS, R
5	Monoterpene, M^+ 136 *m/z* 93 (100), 41 (47), 79 (45), 39 (30), 69 (25), 67 (24)	7	MS
6	Myrcene	241	MS, R
7	α-Phellandrene	3	MS, R
8	Limonene	38	MS, R
9	β-Phellandrene	126	MS, R
10	1,8-Cineol	35	MS, R
11	*Z*-β-Ocimene	449	MS, R
12	*E*-β-Ocimene	99	MS, R
13	Unknown, *m/z* 41, 69, 67, 43, 55, 93	3	
14	Allo-ocimene	5	MS, R
15	M^+ 93, fragment from linalool? *m/z* 41 (100), 79 (87), 43 (67), 59 (50), 71 (46), 93 (31)	5	MS
16	β-Thujone	5	MS, R
17	Unknown, M^+ 136 *m/z* 79 (100), 43 (68), 81 (64), 41 (62), 93 (58), 39 (52), 77 (38)	12	
18	Pinocamphone	20	MS
19	M^+ 150, monoterpene ketone? *m/z* 53 (100), 81 (81), 108 (55), 41 (66), 107 (32), 39 (30)	171	MS
20	Linalool	102	MS, R
21	Methyl citronellate	9	MS, R
22	Unknown, M^+ 150 *m/z* 83 (100), 55 (54), 39 (17), 41 (7), 53 (6), 84 (5)	25	
23	α-Guaiene	167	MS, R
24	M^+ 109 *m/z* 109 (100), 83, 55, 41, 95, 67, 109	7	
25	Pinocarveol, M^+ 119 *m/z* 41 (100), 55, 92, 91, 70, 83, 43	12	MS
26	α-Humulene	12	MS, R
27	Monoterpene, M^+ 134 *m/z* 91 (100), 41 (61), 119 (51), 92 (48), 43 (30), 109 (30), 77 (26)	2	MS
28	Verbenone, M^+ 150 *m/z* 107 (100), 39 (86), 80 (85), 41 (82), 135 (76), 91 (72), 79 (55)	26	MS
29	Germacrene D	76	MS, R
30	α-Terpineol	62	MS, R
31	Bicyclogermacrene	25	MS, R
32	*E,E*-α-Farnesene	10	MS, R
33	Myrtenol	25	MS, R
34	Germacrene B	21	MS, R
35	Carvylacetate I	10	MS
36	Carvylacetate II	2	MS
37	Eugenol methyl ether	8	MS, R
38	*E*-Nerolidol	11	MS, R
39	Eugenol	39	MS, R
40	Isoeugenol methyl ether	18	MS, R
41	Isoeugenol	86	MS, R

[+] Means of identification were: MS, comparison of mass spectra with MS data system; R, comparison of GC-retention times with authentic reference compounds.

The total yield of essential oil was 0.24% (g/g) of the fresh weight.

Cell culture. Heterotrophic and photomixotrophic callus cultures on media SH, SH 1, SH 2 and the heterotrophic culture on medium SH 3 contained structurally simple compounds as the main volatile constituents: primary alcohols, acetic acid, acetoin, etc. No volatile terpenes could be detected in these cultures.

First indications for oil production of the green cell culture on medium SH 3 were derived from sensory evaluations. This cell line had a spicy odour reminiscent of fresh pepper and distinctly differing from the smell of the parent plant.

GLC-MS analysis confirmed the presence of mono- and sesquiterpenes in the callus extract. The composition of the essential oil of a 48 days old cell culture is shown in Table II. 11 monoter-

Table II. Compounds identified in the essential oil of photomixotrophic callus cultures of *Coleonema album*.

Peak No.	Compound	Quantity [mg/kg fr. w.]	Identification method
1	Ethyl 2-methylbutanoate	1.7	MS, R
2	α-Pinene	0.5	MS, R
3	β-Pinene	0.1	MS, R
4	Sabinene	0.2	MS, R
5	Monoterpene, M^+ 136 *m/z* 93 (100), 41 (47), 79 (45), 39 (30), 69 (25), 67 (24)	0.1	MS
6	Myrcene	1.5	MS, R
7	α-Phellandrene	1.7	MS, R
8	Limonene	3.4	MS, R
9	β-Phellandrene	23.9	MS, R
11	β-Ocimene	0.1	MS, R
23	α-Guaiene	0.9	MS, R
31	Bicyclogermacrene	1.4	MS, R
34	Germacrene B	0.6	MS, R
42	δ-3-Carene	0.1	MS, R
43	α-Terpinene	0.1	MS, R
44	*p*-Cymene	0.1	MS, R
45	Cycloalkene, $C_{11}H_{16}$ *m/z* 79 (100), 94 (31), 77 (18), 91 (17), 39 (16), 105 (15)	1.2	MS
46	δ-Elemene	0.6	MS, R
47	Cycloalkene, *m/z* 79 (100), 94 (42), 77 (28), 91 (19), 39 (18), 105 (15)	1.7	MS
48	δ-Selinene *m/z* 161 (100), 105 (93), 204 (73), 91 (72), 119 (69), 189 (66)	5.7	MS
49	δ-Guaiene	0.1	MS, R
50	Unknown, *m/z* 41 (100), 44 (63), 68 (61), 67 (56), 81 (41)	0.1	
51	α-Elemene *m/z* 121 (100), 93 (88), 41 (64), 40 (63), 39 (62), 136 (43)	0.1	MS
52	Unknown, *m/z* 141 (100), 41 (27), 42 (25), 55 (13), 113 (12), 59 (10)	0.1	
53	Sesquiterpene alcohol? *m/z* 81 (100), 161 (90), 43 (89), 105 (70), 119 (48), 91 (43)	1.7	MS
54	Unknown, *m/z* 43 (100), 71 (32), 41 (31), 55 (29), 67 (17), 97 (16)	8.1	
55	Unknown, *m/z* 43 (100), 67 (50), 41 (35), 81 (44), 55 (27), 54 (23)	4.1	

[+] Means of identification were: MS, comparison of mass spectra with MS data system; R, comparison of GC-retention times with authentic reference compounds.

penes and 5 sesquiterpenes could be identified. Main constituents were the hydrocarbons β-phellandrene, δ-selinene and the unknown compound **54.** Sniffing analysis revealed that **54** was responsible for the characteristic peppery odour. This component, which could not be detected in the parent plant extract, and several other constituents require further structural elucidation. Monoterpene **5** could be identified in both the plant and the cell culture. Its fragmentation pattern indicates a menthadien skeleton. The compounds **45** and **47** are cycloalkene derivatives. Comparing the fragmentation patterns of **47** and δ-elemene, **47** might represent a closely related structure. From the mass spectra of compounds **50, 52, 54,** and **55** a precise information on the chemical class is not available. The total yield of essential oil was 0.006% of the fresh weight. Production of flavour compounds has been observed for now at least 9 months.

The development of a well-growing suspension culture from freshly established calli posed experimental problems. Finally, a well-growing photomixotrophic suspension was obtained. The suspension culture exhibited the same typical odour characteristics as the callus culture when grown under the same nutrient (SH 3) and light conditions.

Comparison of the essential oil of green parts of the plant and of the mixotrophic cell culture

The pattern of essential oil constituents showed significant qualitative and quantitative differences:

Thirteen constituents of the plant were also produced by callus cells: ethyl 2-methylbutanoate, α- and β-pinene, sabinene, myrcene, α- and β-phellandrene, limonene, *Z*-β-ocimene, monoterpene **5,** and the sesquiterpenes α-guaiene, bicyclogermacrene, germacrene B.

Z-β-Ocimene is the main compound of the plant essential oil, whereas in cell culture β-phellandrene is the major constituent. α- and β-phellandrene are main compounds in the essential oil of pepper [17]. As the aroma quality of the callus volatiles resembled that of pepper, the extract was supposed to contain other typical pepper constituents, such as the caryophyllenes. However, none of the unidentified callus oil components could be attached to known pepper constituents.

The pinane derivatives (except α- and β-pinene), the oxygen containing monoterpenes and the following volatiles of the plant were not present in cell culture extracts: allo-ocimene, *E*-β-ocimene, 1,8-cineol, *E*-nerolidol, *E,E*-farnesene, α-humulene, germacrene D, the phenylpropanoids and the unidentified compounds.

The cell culture accumulated some volatiles which were not detectable in the plant: δ-3-carene, *p*-cymene, α-terpinene, the sesquiterpenes δ-elemene, δ-guaiene and δ-selinene, the "impact" compound **54** and the remaining unidentified products.

Ultrastructural features

Examination of the ultrastructure of the terpene-producing callus cells revealed that they contained a heterogeneous population of plastids. Upon illumination, chloroplast differentiation occurred. In all chloroplasts thylakoids were arranged mainly in grana (Fig. 2). Most of the chloroplasts contained significant amounts of starch. Besides these plastids, different leucoplast forms (characterized by the absence of plastoribosomes and thylakoids) were observed, some containing starch grains, some containing osmiophilic droplets (Fig. 3–5). Chloroplasts and leucoplasts were not associated in the same cell. Thus, it is supposed that there exist at least two different kinds of cells in this culture, which are characterized by different plastid forms.

In heterotrophic callus cultures growing on the same medium variant, no plastids could be visualized (Fig. not shown). An explanation may be that these cells contained small proplastids with little structural organization.

Discussion

Coleonema album (Thunb.) Bartl. *et* Wendl, is a small rutaceous shrub native to South Africa. Previous chemical studies upon this plant were limited to the study of limonoid bitter principles [18]. Of the closely related *C. pulchellum,* an investigation of the volatile oil has been reported recently [19]. Major volatiles of *C. pulchellum* that are common to both species are: α-pinene, β-pinene, myrcene, β-phellandrene, and germacrene D.

In contrast to most of the earlier reports on *in vitro* cultures of essential oil plants, tissue cultures of *C. album* when cultivated under appropriate conditions are a rich source of volatile secondary

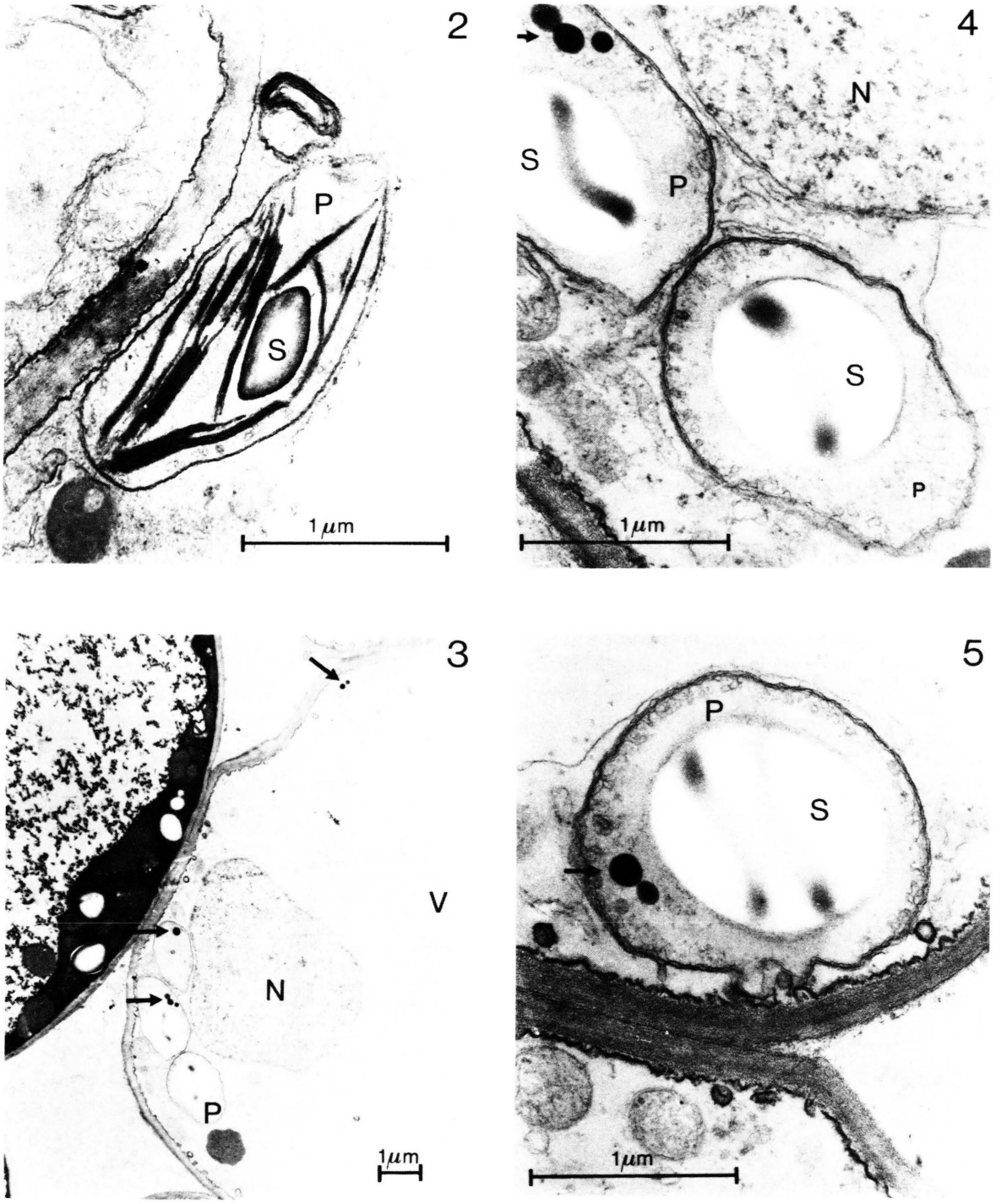

Fig. 2–5. Photomixotrophic calli of *Coleonema album* grown on SH 3 medium. Abbreviations: n, nucleus; p, plastid; s, starch grain; v, vacuole; Fig. 2 chloroplast (enlargement × 44,100); Fig. 3 cell with leucoplasts and osmiophilic droplets (arrows; enlargements × 8790); Fig. 4 and 5 leucoplasts (enlargement × 44,100) and osmiophilic droplets (arrows).

metabolites. The absolute concentrations of volatiles in the callus culture were lower than those accumulated by the plant, but the estimated productivities (time of accumulation in cell culture: 4 weeks *vs.* plant: 30 weeks) of both sources differed by a factor of about 5 only. In the case of *C. album,* illumination and distinct growth factor conditions were a prerequisite. It is generally assumed that some correlation exists between the degree of cell differentiation and the level of accumulation of volatiles [20–22]. The importance of chloroplast differentiation for the formation of secondary products has been demonstrated in various tissue cultures in which secondary metabolites were induced after greening of the cells [23]. Common biosynthetic routes can be formulated for terpenoid flavour compounds and the phytyl side chain of chlorophylls. In chloroplasts, several light-dependent enzymes of isoprenoid biosynthesis were localized [24].

The results with the photomixotrophic cell lines on original SH-medium and on media with different growth factor concentrations show that light is a necessary, but not the only factor for secondary product formation. Accumulation markedly depends on the levels of hormones present in the medium. The comparison of the productivity on media SH 2 and SH 3 demonstrates that not only the ratio of concentrations but also the absolute amounts of the growth factors are important. Both media have the same hormone ratio, but only the elevated hormone concentrations favoured terpenoid production.

Thus, in *C. album* cell culture, at least two conditions have to be met for secondary product accumulation to occur. In addition to illumination, further biochemical and morphological differentiations have to be induced by changing the hormone levels. Possibly, the biosynthetic ability of the photomixotrophic callus on medium SH 3 is associated with the development of characteristic leucoplasts in the cells. The involvement of these organelles in monoterpene biosynthesis was demonstrated using isolated leucoplasts of *Citrofortunella mitis* [25]. In reports with similar electron micrographs, osmiophilic droplets within leucoplasts were assumed to represent terpenes [26]. According to these data, heterotrophic *C. album* cultures on SH 3 medium contained neither leucoplasts nor terpenoid constituents.

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