Accumulation of Chlorophyll and Essential Oils in Photomixotrophic Cell Cultures of *Citrus* sp.

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Z. Naturforsch. **51c,** 657–666 (1996); received March 12/June 18, 1996

Citrus, Cell Culture, Photomixotrophy, Oligoprenoids, Limonene

Heterotrophically or photomixotrophically initiated callus cultures *of Citrus paradisi*, *C. limon* and *C.aurantifolia* were grown on different nutrient media and under different light regimes. Calli of *C.paradisi* that contained > 140 mg chlorophyll per kg wet weight accumulated about 40 volatile mono- and sesquiterpene hydrocarbons, oxigenated terpenes and aliphatic aldehydes. Upon five subcultivations the best yielding callus contained about 5% (186 mg × kg^{-1} wet wt) of the volatiles found in peel tissue (exo/mesocarp section), and about the twentyfold amount of that found in the fleshy endocarp. The composition of the essential oils from most of the cell cultures equalled grapefruit peel oil, but was shifted to a more fruit flesh-like composition, after the concentration of gellan gum in the medium was increased from 3 to 9 g per L. *C. limon* produced 11 monoterpenes and n-nonanal (40 mg × kg^{-1} wet wt max.), and *C.aurantifolia* yielded limonene only (4.4 mg × kg^{-1} wet wt max.). For all of the indicated species chlorophyll content and accumulation of volatiles were positively correlated. Addition of exogenous valencene to suspended cells of *C.paradisi* led to a stable concentration of the conversion product nootkatone. This stably maintained level suggested that a decreased catabolism of available carbon sources might have accounted for the significant accumulation of essential oil constituents.

Introduction

In view of the wide usage of *Citrus* species in the food, pharmaceutical and cosmetic industries, it is not surprising that cell cultures were established to investigate somatic embryogenesis and hybridization, micropropagation, gene transfer and flavonoid formation (Del Rio and Ortuño, 1994). Volatile constituents have received less attention, as it was shown that *Citrus* cultures, like most other cell cultures derived from essential oil plants, do not accumulate anything like significant levels (Bricout and Paupardin, 1974; Paupardin, 1974; Drawert and Berger, 1982).

More recently, dark grown callus cultures of various *Citrus* species were reported to produce valencene and nootkatone (0.1 and 1.6 mg × kg^{-1} wet wt max., respectively) when grown on a 10% orange juice medium (Del Rio and Ortuño, 1994). The maximum values for valencene were found in young calli, whereas nootkatone accumulation started in older calli and was accompanied by the generation of lysigenous intercellular spaces. Calli of *C.aurantifolia* S. produced a total of 2.8 g volatile oxygenated terpenes × kg^{-1} wet weight, when grown on an optimized medium under a 12 h photoperiod (Agrawal *et al.*, 1991). These authors successfully used the central composite rotatory design, a statistical tool for the rapid optimization of the growth medium, to improve the chemical environment of the cultured cells and identified citral, terpinyl and amyl acetate, dodecanal and a sesquiterpene alcohol as the volatile constituents. No attempts have ever been made to quantify the effect of light on the synthesis of volatile metabolites in *Citrus* cell cultures.

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Materials and Methods

Cell cultures

Citrus paradisi Macf. cv. White Marsh, *C. limon* (L.) Burm.f. and *C.aurantifolia* Swingle fruits were from the local market. Intact, mature fruits were

surface sterilized with 70% ethanol (one min), followed by 2% w/v $Ca(OCl)_2$ solution for 10 min and then rinsed three times with sterile water. Callus was initiated using excised flavedo and MS (Murashige and Skoog, 1962) and a modified MS medium (MSL), as applied by Agrawal *et al.* (1991) for the production of terpenes. This medium differed from the known MS medium in these constituents: KNO_3 1245 $mg \cdot l^{-1}$, KH_2PO_4 1500 $mg \cdot l^{-1}$, and phytoeffectors (Sigma) as indicated in the text. The solidifier was 3 g of gellan gum (Kelco) per l for each medium. The calli initiated on MSL grew only slowly when subcultured on other media. The SH4 variant, developed during an earlier study (Berger *et al.*, 1990), differed from the original SH medium (Schenk and Hildebrandt, 1972) in the contents of sucrose (10 $g \cdot l^{-1}$), lactose $\times H_2O$ (10 $g \cdot l^{-1}$), kinetin (2 $mg \cdot l^{-1}$), and *p*-chlorophenoxyacetic acid (none). The explants and all subcultures were maintained under continuous light of a Hg-high pressure lamp (Osram HQL/R 80 W, 3000 lx = 3000 $cd \cdot sr \cdot m^{-2}$), or under the light of a neon tube (colour 12 with λ_{max} = 435 and 545 nm, Osram) and at 23 °C.

For the sake of comparison, cells were cultured under or reconverted to heterotrophic conditions in the dark at 27 °C. Suspended cells were kept under identical conditions at 140 rpm in 300 ml Erlenmeyer flasks. Inoculation density was 10 g filtered wet wt · 100 ml^{-1} medium.

Fully developed primary callus was transferred to cultivation conditions as indicated in Tables I and III, and analyzed immediately and after the fifth subcultivation cycle. In order to evaluate the effects of light colour, photoperiod, concentration of gelling agent and phytoeffectors on growth and accumulation of secondary products, one set of experimental combinations was designed for each of the species: The light colours and the gellan gum concentrations used did significantly stimulate chlorophyll formation in concurrent experiments with other members of the Rutaceae family (data not shown); phytoeffector concentrations were adopted from a previous study on *Citrus* (Agrawal *et al.*, 1991) and a second variant with increased concentrations included. Callus cells were transferred to fresh medium every 2 to 7 weeks, and suspension cells every 3 weeks.

All chemicals used for preparation of nutrient media and chemical analysis were p.a. grade (Merck); water was deionized and showed a conductivity of < 0.2 μS.

Determination of chlorophyll, growth index and light intensity

The sum of chlorophyll *a* and *b* was determined spectrophotometrically at 652 nm in a 80% (v/v) acetone extract using 5 g wet wt. The ratio of chlorophyll *a/b* was determined according to Ziegler and Egle (1965).

The growth index was calculated by comparing cell wet masses at harvest and at inoculation as $(M_h - M_i)/M_i$.

Light intensity was measured on surface cell level using a Metrux lux meter (Metrawatt) and a Data Logger LI-1000, I-COR with quantum sensor (PAR). 1 lx = 1 $lm \cdot m^2$, 1 lm = 1 candela · steradiant.

Addition of valencene

A single addition of valencene in ethanol (one mL of a stock solution 1 $mmol \cdot l^{-1}$ to 150 ml of medium) was made at the end of the linear growth phase. Purity (determined by GLC) was > 99.8%.

Preparation of the extracts for GLC analysis

Extracts from the fruit parts of the plant and from callus or suspension cultures were prepared immediately after buying or harvesting of the plant material. Callus cells were carefully separated from adhering medium, mixed with the twofold amount per weight of MeOH and 1 ml internal standard (100 μg 2-undecanone · ml^{-1}) in a cooled Waring Blendor (30 s). The homogenate was centrifuged (5000×*g*, 10 min, -4 °C), and 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, and subjected to capillary gas chromatography or mass spectroscopy.

Gas liquid chromatography and coupled mass spectrometry

A 25 m × 0.32 mm i.d. fused silica capillary column coated with SE 54, 1μm film thickness (Leupold, Weihenstephan) was used. Conditions: Tem-

perature program 50 °C for 5 min isothermal, then at a rate of 3 °min^{-1} to 250 °C; carrier gas hydrogen, 1.5 ml min^{-1} (105 °C); injector split ratio 1:15 or splitless; injector temperature 250 °C, detector temperatur 260 °C. The quantitative evaluation referred to the internal standard using an electronic integrator (HP 3396).

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. The chromatographic conditions were identical, but carrier gas was helium at 1.2 ml·min^{-1} (60 °C). Spectra were analyzed either by using reference compounds of the collection of the institute or by comparison with an internal NBS-library (ca. 36000 spectra).

All quantitative data originate from duplicate experiments using the same inoculum and are given as a mean value. If the deviation of the results was > 10%, the experiment was repeated. Further subculturing did not significantly affect the quantitative data. None of the volatiles indicated in Table II was present in freshly prepared media.

Results

Cell cultures

The growth of photoheterotrophic calli of *C.paradisi* was slow and reached indices of 3 to 5 on the different media. The very firm aggregates had diameters of < 5 cm. Photomixotrophic calli showed similar growth indices, but were softer, dark green, and formed smaller aggregates (< 1 cm). Conversion of photoheterotrophic calli to photomixotrophic ones did not result in chlorophyll formation, apart from MSL medium (< 15 $\mu g \times g^{-1}$). Mixotrophic cells contained 7 to 9 wall associated chloroplasts, irrespective of the actual chlorophyll content. Thorough light microscopic investigation gave no hints on the presence of secretory cavities or idioblasts, oil blisters, trichomes or ducts, but showed a heterogeneous morphology with interspersed differentiation including tracheidal and red coloured cells with irregular, evaginated walls. Formation of rootlets or stems was not observed.

Primary callus of *C.limon* was observed after 4 weeks under heterotrophic and after 9 weeks under phototrophic conditions. The growth index of the soft heterotrophic cells was 9 to 10, while firm, aggregating (< 4 mm) phototrophic cells grew with an index of 6 to 7. Conversion of heterotrophically initiated cells to the phototrophic state failed on all media. Phototrophic cells with different chlorophyll contents constantly contained 3 to 5 chloroplasts and sometimes fragments of tracheidal wall structures. No other cyto- or organ differentiation was found.

Flavedo explants of *C.aurantifolia* callused after 2 and 6 weeks under heterotrophic and phototrophic conditions, with growth indices of 9 to 10 and 5 to 6, respectively. The heterotrophic cells were soft and crumbly, whereas the phototrophic ones were yellow-green to dark green and aggregated.

Chemical analysis of constituents of C.paradisi

Total chlorophyll and chlorophyll *a/b* ratios reached stable values after five subcultivations of *C.paradisi* under 3,000 lux (Table I). Chlorophyll formation occurred in all illuminated cells on all nutrient media. A combination of the MSL medium, light colour 12, 24 h photoperiod and the twofold phytohormone concentrations as compared with the original medium yielded maximum amounts. As the calli grown on MSL medium exhibited a typical *Citrus* aroma, these cells were examined for their volatile composition.

Volatiles in fruit and cultured cells of C.paradisi

Citrus essential oils are complex mixtures of oligoisoprenoids and fatty acid degradation products. The sensory impression is determined by (+)-*4R*-limonene, and quantity and ratio of side and trace components impart species character. Composition and yield of volatile constituents in *C.paradisi* cell cultures growing under different chemical and physical conditions were compared with the essential oil of the fruit of the plant. GLC-MS examination of the essential oil of *C.paradisi* reconfirmed the presence of 49 constituents, mainly monoterpenes, in the flavedo/albedo (exocarp) and edible (mesocarp/endocarp) portion of the fruit oil (Wilson and Shaw, 1987). Limonene, the precursor of the oxygenated p-menthane structures, accounted for 88% of the volatile fraction in the peel, fol-

Table I. Chlorophyll accumulation (mg × kg^{-1} fresh wt) in hetero- and photomixotrophically initiated callus of *C.paradisi*.

Exp no.	Medium	Light colour	Photoperiod light/dark(h)	Gellan gum (g × l^{-1})	Phytoeffector[a] conc. (mg × l^{-1}) 2,4-D	NAA	IAA	IBA	Kin	Chlorophyll	Chlorophyll *a/b*
heterotrophic											
	SH[b]	HQL	24/0	3	0.5					n.d.	–
	SH4	HQL	24/0	3		0.5			2.0	n.d.	–
	MS[c]	HQL	24/0	3	1.0				0.1	n.d.	–
	MSL	HQL	24/0	3		0.25	1.0	0.5	0.25	15	1.4
photomixotrophic											
	SH	HQL	24/0	3	0.5					35	1.4
	MS	HQL	24/0	3	1.0				0.1	47	1.4
	SH4	HQL	24/0	3		0.5			2.0	73	1.5
1	MSL	HQL	16/8	3	0.25	1.0	0.5	0.25	142	1.9	
2	MSL	HQL	24/0	3		0.25	1.0	0.5	0.25	180	1.9
3	MSL	12	16/8	3		0.25	1.0	0.5	0.25	161	1.8
4	MSL	12	24/0	3		0.25	1.0	0.5	0.25	193	2.0
5	MSL	12	16/8	3		0.5	2.0	1.0	1.0	243	2.7
6	MSL	12	24/0	3		0.5	2.0	1.0	1.0	**310**	2.9
7	MSL	12	24/0	9		0.25	1.0	0.5	0.25	191	2.2
8	MSL	HQL	24/0	9		0.25	1.0	0.5	0.25	183	2.2
9	MSL	12	24/0	9		0.5	2.0	1.0	1.0	212	2.5

[a] 2,4-dichlorophenoxyacetic acid; naphthylacetic acid; indoleacetic acid; indolebutanoic acid; kinetin.
[b] SH medium additionally contains p-chlorophenoxyacetic acid (2.0 mg × l^{-1}).
[c] MS medium additionally contains 6-benzylaminopurine (0.1 mg × l^{-1}).

lowed by myrcene (2%), n-octanal (2%) and citronellal (1%). (2*E*)-hexenal and nootkatone dominated in the fleshy part. When callus was grown under the conditions of experiment No. 4 (Table I), the extracted volatiles comprised thirty-eight compounds. Chemical structures and quantitative composition were very similar to cold pressed grapefruit peel oil (Table II). All components extracted from the cell culture were also found in the peel oil, except tri- and tetradecanal. Some trace constituents of the peel were not found in the cell culture, probably as a result of a lack of analytical sensitivity. Experiment No. 6 (Tables I and II) resulted in maximum formation of limonene and total volatiles, but at the expense of the diversity of the chemical composition. These accumulations represent de novo synthesis, because

– carry-over from the primary callus that contained only traces of volatiles was negligible,
– pattern and quantity of volatiles reproducibly depended on the subcultivation conditions chosen and changed upon variation of the nutrients, and
– accumulation dropped to zero in suspension cultures and was restored on subsequent transfer to solidified medium.

Fig. 1 shows distinct quantitative differences in extracts of the tissues from the intact plant and of the best yielding photomixotrophic callus culture: The cell culture contained about 5% (186 mg × kg^{-1} wet wt) of the volatiles found in peel tissue (exo/mesocarp section), and about the twentyfold amount of that found in the fleshy endocarp. The direct comparison of the aroma producing potentials of differentiated plant part and cell culture is complicated by the heterogenous morphology and concentration gradients of the fruit. If the whole fruit is regarded as a source of aroma, the wet weight based figures of differentiated tissue and cell culture are in the same order of magnitude (0.029 vs. 0.019% w/w); however, on a time base,

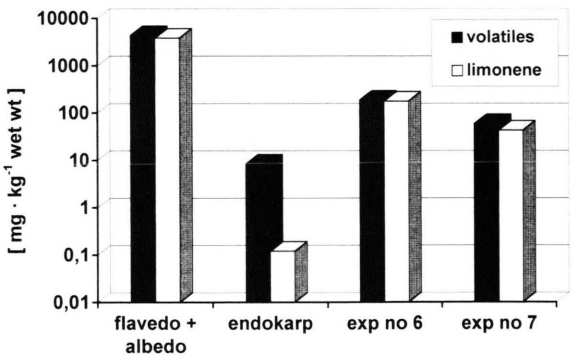

Fig. 1. Total volatiles and limonene in differentiated tissues and photomixotrophic callus of *C.paradisi*.

Table II. Volatiles in photomixotrophic callus of *C.paradisi* (conds see Table I).

Constituent	Experiment No. 4 (mg × kg^{-1} fresh wt)	Experiment No. 6	Method of identification
α-Pinene	0.09	0.58	MS, RT
β-Pinene	trace	trace	MS, RT
Sabinene	0.22	0.67	MS, RT
Myrcene	0.74	3.75	MS, RT
(+)-Limonene	48.35	176.73	MS, RT
(*E*)-β-Ocimene	0.06	0.28	MS, RT
n-Octanal	1.17	0.41	MS, RT
(3*Z*)-Hexenal	0.11		MS
n-Nonanal	0.41		MS, RT
Limonen-1,2-epoxide	0.02		MS
(*Z*)-Linalool oxide	0.02		MS
α-Pinene oxide	0.05		MS
(*E*)-Sabinene hydrate	0.20	0.32	MS, RT
Citronellal	0.95	0.59	MS, RT
α-Copaene	0.02	0.16	MS
n-Decanal	0.35	0.23	MS, RT
Linalool	0.07	0.16	MS, RT
n-Octanol	0.08	0.65	MS, RT
β-Caryophyllene	0.19		MS, RT
Terpinen-4-ol	0.24		MS, RT
(*E*)-2,8-p-Menthadien-1-ol	0.03		MS, RT
α-Humulene		0.08	MS, RT
Neral	0.24	0.15	MS, RT
(*E*)-Piperitol	0.04	0.17	MS
α-Terpineol	0.22	0.27	MS, RT
n-Dodecanal	0.76	0.17	MS, RT
Geranial	0.29	0.17	MS; RT
δ-Cadinene		0.19	MS; RT
Geranyl acetate	0.03	0.16	MS; RT
Perillaldehyd	0.05		MS
Nerol	0.03		MS; RT
n-Tridecanal	0.20		MS; RT
(*E*)-Carveol	0.03		MS; RT
Geraniol	0.04		MS; RT
n-Tetradecanal	0.20		MS; RT
Nerolidol	0.06		MS; RT
Nootkatone	0.30	0.64	MS, RT

the productivity of the cultured cells is superior (13 months from anthesis to mature fruit vs. 4 weeks of subcultivation). Permanent illumination instead of a 16/8 hours photoperiod stimulated the formation of volatiles, as did the use of light colour 12 neon tubes. A 50% increase of total auxin and kinetin concentrations was lethal.

Changed conditions of cultivation also affected the pattern of volatiles (Fig. 2). Experiments No. 1 to 6 resulted in essential oils with a composition that closely resembled that of peel oil. Increased concentration of the gelling agent in experiments No. 7 to 9 reduced the diffusion of nutrients into the cells and the secretion of volatiles into the medium. In turn, the saturated aldehydes from six

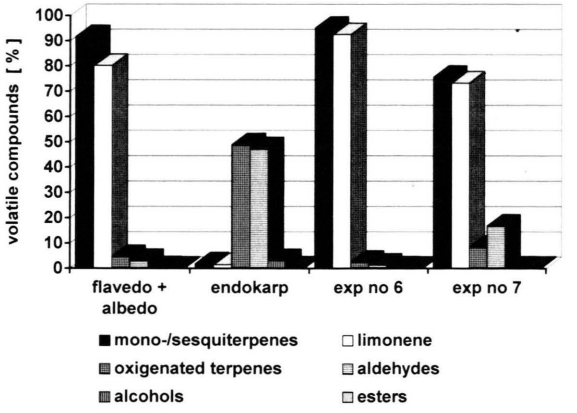

Fig. 2. Composition of essential oils in differentiated tissues and photomixotrophic callus cultures of *C.paradisi*.

to fourteen carbons increased. In contrast to fruit essential oil, limonene remained the major compound in these cell cultures. Formation of volatiles was not observed in any of the heterotrophically initiated cells nor in phototrophic cells that contained less than 140 mg chlorophyll per kg.

A linear regression curve resulted from the correlation of the contents of total volatiles and chlorophyll, as obtained from experiments no. 1 to 9 (Fig. 3). Smaller amounts (< 10%) of volatiles leaked into the surrounding agar zones, but were not included into these calculations. The worser fit of the regression line for experiments no. 5 and 6 may be interpreted as a declining slope passing over to a saturation curve-type behaviour; however, a further systematic approach to improve terpene yields was not in the scope of this work. Production of aroma compounds has been observed for more than 2 years of continued sub-culturing.

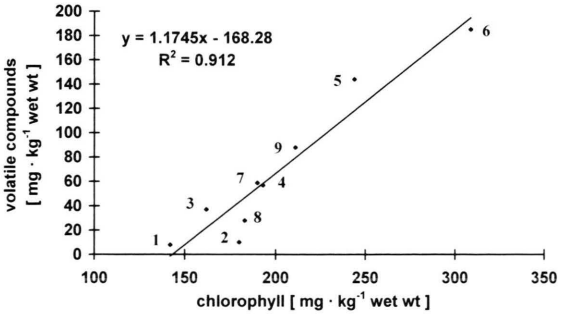

Fig. 3. Chlorophyll *vs.* total volatiles in photomixotrophic callus cultures of *C.paradisi*.

Incubation of suspended, stationary phase cells with exogenous valencene led to the intermediary formation of the 2-hydroxy-derivative, followed by conversion to the 2-oxo-compound, nootkatone (68% in 24 h). The once reached concentration of nootkatone (ca. 0.7 mg × l^{-1}) was maintained for another 48 h without noticable change. Then the experiment was terminated. The competing chemical oxidation was not measurable under conditions.

Volatiles in fruit and cultured cells of C.limon

The same chemical and light conditions that favoured terpene formation in *C.paradisi* cells were successful for *C.limon* (Table III). Because of the about fivefold lower actual concentrations, only 12 volatiles were identified, among them acyclic, mono-, and bicyclic monoterpene hydrocarbons and oxygenated derivatives. Limonene was again the major volatile, and a fairly constant ratio of limonene/β-pinene of 5.0 ± 0.4 was found. According to Bauer and Garbe (1985), cold pressed lemon oil is composed of 65% limonene, 8 to 10% β-pinene, 8 to 10% γ-terpinene, and 3 to 10% neral/geranial (= citral, the character impact component). A comparison with the concentration of volatiles accumulated by the cell cultures (Table III) shows that the lipophilic extracts equal the reported composition of lemon essential oil. The yields were again positively affected by continuous light, light colour 12, and by elevated concentrations of gelling agent and phytoeffectors.

Other analogies between *C.limon* and *C.paradisi* were the correlation of chlorophyll and terpene contents (Fig. 4) and the complete absence of volatiles in suspension cultures. When suspended, light grown cells were transferred back onto solid medium, the capability to form volatiles was fully restored after 3 subcultivations.

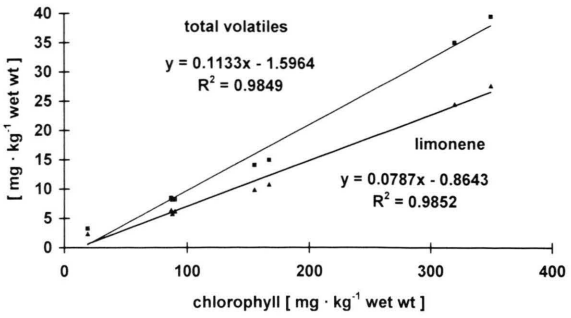

Fig. 4. As in Fig. 3 for *C.limon*; top line: total volatiles, lower line: limonene.

Volatiles in cultured cells of C.aurantifolia

Duplicating the experimental conditions as given in Table III, the experiments were repeated with lime cells (Fig. 5). A significantly faster growth was associated with an eightfold lower actual accumulation of volatiles as compared to *C.limon*. Limonene was the only volatile identified. Chlorophyll and limonene formation were again closely correlated, and the same qualitative relation of cultivation conditions and chlorophyll/ limonene accumulation was found. The course of the regression curve fitted best with a typical exponential saturation curve; this difference may be attributed to the lower over-all productivity. A maximum of 390 mg chlorophyll and of 4.4 mg

Table III. Accumulation of chlorophyll (mg × kg^{-1} fresh wt) and volatiles (μg × 100 g^{-1} fresh wt) in photomixotrophically initiated callus of *C.limon* (MSL medium, 3,000 lx continuous light, 4 weeks subcultivated).

Exp No.	Light color	Gellan gum (g × l^{-1})	Phytoeffector[a] (mg × l^{-1}) NAA	IAA	IBA	Kin	Chlorophyll	β-Pinene	Limonene	Total volatiles
1	HQL	3	0.25	1.0	0.5	0.25	19	49	234	326
2	HQL	3	0.5	2.0	1.0	1.0	88	108	574	817
3	HQL	9	0.25	1.0	0.5	0.25	90	114	617	817
4	HQL	9	0.5	2.0	1.0	1.0	87	114	637	846
5	12	3	0.25	1.0	0.5	0.25	155	204	983	1,409
6	12	3	0.5	2.0	1.0	1.0	167	222	1,075	1,497
7	12	9	0.25	1.0	0.5	0.25	320	505	2,460	3,510
8	12	9	0.5	2.0	1.0	1.0	**350**	602	2,776	3,958

[a] see Table I.

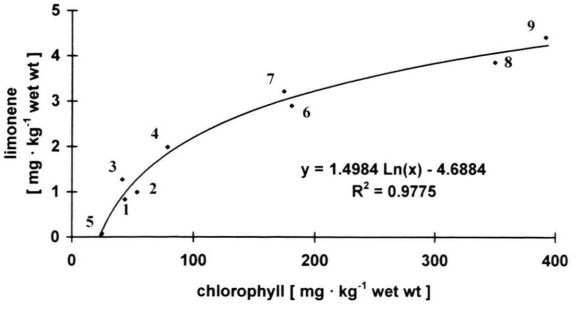

Fig. 5. Chlorophyll *vs.* limonene in photomixotrophic callus cultures of *C.aurantifolia.*

limonene per kg wet weight, respectively, was reached using the conditions No. 8 of Table III (exp. No. 9 in Fig. 5).

Discussion

The effect of light

Lower oligoprenoids are important as insect repellents, antifeedants and as chemical messengers in ecological interactions in general. Many observations with cultures of essential oil plants indicated, however, that cells in a sterile and confined environment produce only very low amounts of terpenoids or lose accumulation completely. Various attempts to overcome this situation included improving the availability of the early precursor by feeding mevalonate (Nabeta *et al.*, 1993), lowering the cultivation temperature (Chae and Park, 1994), and changing the nutrient composition (Agrawal *et al.*, 1991).

Both the quality and quantity of light affect the synthesis of terpenes in intact plants by regulating the key enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (Stermer *et al.*, 1994). The photoperiod received by leaves of *Mentha piperita* directly influenced the oil composition (Voirin *et al.*, 1990). Similarly, the level of terpene accumulation was linked to the photonic fluence in *Ginkgo biloba* leaves (Flesch *et al.*, 1992). An increase in the levels of chloroplast isopentenyl diphosphate (IDP) isomerase during light-stimulated carotenoid biosynthesis in maize has been observed (Albrecht and Sandmann, 1994).

Analogous reports on cell cultures referred to the formation of root-like volatiles in heterotrophic and leaf-like volatiles in photomixotrophic suspension cells of *Ruta graveolens* (Jordan *et al.*, 1986), and the formation of leaf-like terpenoids in photomixotrophic callus of *Coleonema album* (Berger *et al.*, 1990). In geranium cultures, a stimulation in the accumulation of essential oil of some 200-fold was induced by a transfer into a long-light photoperiod (Charlwood *et al.*, 1989). Callus of cotton lavender contained himachalene-type sesquiterpenes when grown under continuous illumination of 600 lux (Banthorpe, 1994). Calli of hop (Banthorpe *et al.*, 1989) and Japanese larch (Nabeta, 1994) were grown under continuous light of 600 and 5,000 lux, respectively, and converted mevalonate precursors into volatile sesquiterpenes. The concentration of a precursor of the norisoprenoid β-damascenone in *Concord* grape callus was raised by light (Shure and Acree, 1994). Electron microscopy of dark grown, valencene and nootkatone producing grapefruit cells revealed that plastids were present, possibly from the light grown preculture (Del Rio and Ortuño, 1994).

Many of the earlier authors did not emphasize the role of light. As a result, light conditions were not changed systematically, and dark grown cells were not compared or not even mentioned, because the respective experiments were regarded „unsuccessful". The data obtained with callus cells of *Citrus* show that the phototrophic state was indispensible for the generation of monoterpenes, and that secondary parameters affected composition and yield. As in most of the studies cited above, suspended cells were inferior in terms of volatile accumulation, and levels of volatiles in the callus did rarely exceed 5% of that found in a specialized tissue of the parent plant.

Plastidic development and terpene formation

The close correlation of chlorophyll and monoterpene accumulation in *Citrus* cells was not found in partially differentiated cultures of *Tanacetum vulgare* (Banthorpe and Wirz-Justice, 1972). When the synthesis of chlorophyll was inhibited these cells still accumulated the same monoterpenes in similar yields to the green cells. It was therefore concluded that monoterpene synthesis was independent of the presence of functional chloroplasts. This apparent contradiction may be solved looking again at intact essential oil plants: Non-photosyn-

thetic secretory cells from glandular trichomes of peppermint were used to demonstrate that the IDP utilized for both monoterpene and sesquiterpene biosynthesis is formed exclusively in leucoplasts which contain all of the enzymes necessary to produce monoterpenes from an exogenous carbon source such as sucrose (McCaskill *et al.*, 1992); intact leucoplasts from *Citrofortunella mitis* converted exogenous IDP into monoterpene hydrocarbons (Gleizes *et al.*, 1987), but only intact chromoplasts achieved the full synthetic potential in the absence of exogenous dimethylallyl diphosphate, the reactive primer molecule (Mettal *et al.*, 1988). This provides evidence that plastids are the subcellular compartment of formation of monoterpenes and diterpenes. Geranyl and geranylgeranyl diphosphate synthases have both been localized in this organelle (Soler *et al.*, 1992; Cheniclet *et al.*, 1992). Oxygenation then takes place at the endoplasmatic reticulum.

Farnesyl diphosphate synthase and the subsequent steps towards the synthesis of sesquiterpenes and triterpenes are localized in the cytoplasm and endoplasmic reticulum. Therefore, IDP formation must proceed along parallel pathways, while the further metabolic fate can change according to developmental requirements. The callus cultures of *Citrus* obviously maintained the physiological state of the tissue from which they were derived (Tables II and III), because both fruit (endocarp) and leaf essential oils show a different composition. A (-)-4*S*-limonene synthase was purified from spearmint, the corresponding cDNA has been isolated, sequenced and the catalytically active enzyme expressed in *E. coli* (Colby *et al.*, 1993). The recombinant limonene cyclase preprotein was catalytically active, producing about the same spectrum and proportions of monoterpene hydrocarbons as the *C.paradisi* callus enzyme (Table II). Though 3 non-identified sesquiterpenes were omitted from Table II, this class of oligoprenoids is, as in peel oil, not well represented in callus extracts. The cytoplasmic sesquiterpene pathway appears to compete poorly for the available IDP relative to the plastidic monoterpene pathway.

Role of phytoeffectors

Phytoeffectors may in some way affect or interact with the processes by which light controls gene expression. Illuminated callus of *Cnidium officinale* produced more volatiles in the presence of naphthylacetic acid (NAA) than 2,4-dichlorophenoxyacetic acid (2,4-D)(Shin and Park, 1994). Similarly, NAA and indoleacetic acid (IAA) promoted the formation of thymol and chlorophyll in calli of *Carum copticum*, while 2,4-D resulted in unorganized proliferation (Prabha *et al.*, 1991). Substitution of 2,4-D and *p*-chlorophenoxyacetic acid by a lower amount of NAA and high kinetin concentrations favoured the formation of essential oil in *Coleonema album* (Berger *et al.*, 1990). The present study achieved maximum product formation by using rather high dosages of a mixture of NAA, IAA, and indolebutanoic acid (Tables I and III).

Application of phytoeffectors may lead to differentiation, organogenesis, and, ultimately, to regenerated plantlets that usually produce the same essential oil as the parent plant (Abou-Mandour *et al.*, 1994; Gozu *et al.*, 1993; Segura and Calvo, 1991)). It was concluded that some degree of specialization must be present in order for both synthesis and accumulation of oligoprenoids to be observed. However, geranium cell lines derived from the same inoculum differed in oil content, but not in morphology indicating that the regulation of these two characters is not linked (Charlwood *et al.*, 1989). In the present study, the number of chloroplasts per cell remained constant under different light and phytoeffector regimes, while concentrations of chlorophyll and terpenes changed. It is supposed that plastid differentiation, i.e. formation and orientation of enzymes and possibly transporters, but not cytodifferentiation or organogenesis is crucial.

Toxicity of oligoisoprenoids

Higher plants excrete mono- and sesquiterpenes into specialized storage sites, such as glandular hairs, lipid vesicles,or resin ducts. In tissue cultures that do not contain an appropriate metabolic sink, essential oil components may severely disturb respiration, photosynthesis, and membrane functions. The toxicity of some monoterpenes to growning cell cultures was determined (Brown *et al.*, 1987). As was estimated accordingly, suspended cells would withstand < 100 mg of certain monoterpenoids per L of medium before toxicity would occur.

More than 1 mg β-damascenone per l (about 5 μM) was toxic to suspended grape cells (Shure and Acree, 1995). Valencene concentrations > 1 mg per l resulted in decreased conversion capacity of *C.paradisi* (Drawert *et al.*, 1984). The strategy of cultured cells to deal with exogenous, toxic terpenes is rapid degradation, a process so prominent that cultured cells have been used for characterizing catabolic pathways (Funk and Croteau, 1993; Berger *et al.*, 1990a). Endogenous accumulation may induce the same catabolic sequences to prevent autotoxicity.

In contrast to earlier results obtained with heterotrophic cultures of many different species, the *C.paradisi* cells accumulated significant amounts of intracellular oligoprenoids (Fig. 1). Though growth rates and oligoprenoid formation were inversely related in all of the experiments shown, only the *C.aurantifolia* cells appeared to reach a saturation level (Fig. 5), while the linearity of the curves in Fig.s 3 and 4 suggests that a further increase of volatiles could occur. The physiological state of photomixotrophic cells may contribute to an improved tolerance to oligoprenoids.

A second aspect may be a reduced access to or activity of endogenous degrading enzymes. This view is supported by the lower tolerance towards exogenous valencene of heterotrophic suspension cultures of *Citrus* (Drawert *et al.*, 1984). These cells reached the maximum nootkatone concentration after 6 h and degraded substrate and product almost completely within 24 h. A remarkably high tolerance towards the monoterpenoid precursor Δ^2-carene and stable accumulation of transformation products has also been reported for illuminated callus cells of *Myrtillocactus geometrizans* (Gil *et al.*, 1994).

Acknowledgements

This work was supported by the Federal Minister of Research and Technology (project no. 0318980A) and by the Fonds der Chemischen Industrie. Valuable advice came from the members of the project group. S. Pilawa and B. Zimmermann are thanked for analytical and technical collaboration.

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