Glycosilation of Terpenols and Aromatic Alcohols by Cell Suspension Cultures of Peppermint (*Mentha piperita* L.)

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Plant cell cultures effectively convert terpenoid and aromatic alcohols into glycosides. A quantitative comparison showed a culture of peppermint *(Mentha piperita)* to be the most active among four species examined. The synthesis of glycosides was affected by the concentration of substrate, incubation period, cell age and aggregation, and by light. Conversion rates of exogenous alcohols were greater than 70% under optimized conditions.

Introduction

The proposal of producing valuable secondary metabolites using plant cell cultures is almost as old as the technique of plant cell suspension culture itself [1]. However, a large number of products of commercial interest accumulates in traces only or not at all in suspension culture [2].

One of the exceptions to the rule is the formation of glycosides of certain secondary metabolites. Some recent examples are the glycosilation of phenolics such as gallic acid [3], coniferyl alcohol [4], *p*-hydroxybenzoic acid [5], flavanones [6], and anthocyanidines [7], as well as the glycosilation of quinones [8], steroids [9], alkaloids [10], and proteins [11].

A few reports only describe the occurrence of glycosidically bound volatile flavours in plant cell cultures. Lang and Hörster [12], investigating cultures of *Ocimum basilicum*, submitted the residue of steam distilled material to enzymic hydrolysis and concluded from the renewed appearance of monoterpenoid and phenylpropanoid compounds in steam distillates that bound forms were present. The accumulation of monoterpene glycosides in cultures of *Vitis vinifera* was demonstrated using [1-^{3}H]geraniol, and 0.6% of the radioactivity applied was recovered after β-glucosidase treatment of a water soluble fraction [13]. The closed system of plant cell culture offers an experimental tool to study the formation of glycosides in order to obtain more insight into the physiological functions of these plant-typical compounds.

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Experimental

Cell cultures

Callus cultures of *Rosmarinus off.* L., induced from seeds, were obtained from Dr. M. Zenk, Munich, and of *Vitis vinifera* cv. Muscat de Frontignan, induced from fruits, were from Dr. C. Ambid, Toulouse. Calli of *Pyrus communis* L., induced from fruits, and of *Mentha piperita* L., induced from leaves, were established at the institute. Suspension cells of rosemary were kept in B5-medium [14], muscat grape in a MS [15] based special medium according to [16], pear in a modified MS-medium [17], and peppermint in SH-medium [18]. White calli were turned into green calli by illumination using a 18 h photoperiod of white light (6000 lux, Osram HQL/R 80 W) at 27 °C. The cell suspension cultures were kept in 300 ml Erlenmeyer flasks with 150 ml medium at 120 rpm, 27 °C, and natural light regime (heterotrophic) or 6000 lux (mixotrophic). After some subcultivations the plant cells were, in contrast to some earlier findings [19], completely devoid of species-typical volatile constituents.

Fractions of the peppermint culture with defined limits of the particle size were obtained by sterile sieving of the suspensions using a stainless-steel sieve (800 μm mesh width) and a filtering screen (400 μm, Monodur PES, Verseidag).

Chemicals

All chemicals were *p.a.* or biochemistry grade except agar No. 1 (Oxoid) and Pectinol C (Röhm).

Aglyca were >98% (g.l.c.) and used as racemates or mixture of isomers unless otherwise indicated. Pure l-menthol (1R, 3R, 4S) and d-menthol (1S, 3S, 4R) was a gift from Dr. R. Emberger, Holzminden. Solvents were redistilled before use.

Determination of aglyca

The cells were carefully separated from adhering medium by filtration and by washing with phosphate buffer (0.15 mol·L^{-1}, pH 5.5), and then mixed with 2 g $NaHCO_3$, the twofold amount per weight of MeOH, and 1 ml internal standard (75 µg isomenthol·ml^{-1}) in a cooled Waring Blendor (30 s). The homogenate was centrifuged (1500×*g*, 10 min, 0 °C), the sediment washed twice and centrifuged, and the supernatants were combined. This solution and, if required the filtered nutrient medium/MeOH-mixture were extracted four times with pentane/diethyl ether (2/1), resulting, with an estimated partition coefficient for menthol of 1.86, in an extraction yield of >98.4%. The concentrated, dried extracts were submitted to capillary gas chromatography as described in detail previously [20].

Determination of glycosides

The remaining solvent-extracted aqueous-methanolic solutions were concentrated *in vacuo* to 10 ml. The concentrate or, if larger yields were expected, an aliquot was further purified by medium pressure preparative RP 18 column chromatography (column 100·12 mm, 12 °C). Hydrophilic contaminants were eluted with 200 ml H_2O, the aqueous fraction discarded, and a glycoside fraction obtained with 250 ml MeOH. The solvent was removed *in vacuo*, the residue dissolved in 20 ml acetate buffer (0.1 mol, pH 4.6) and 200 mg Pectinol C or, for comparison only, β-glucosidase added. For the analysis of the carbohydrate moieties the crude enzyme was purified (t.l.c.) by ultrafiltration (Amicon, excl. s. 10,000 D). Hydrolysis of glycosides was performed in a ground joint vessel for 16 h at 37 °C. Cleavage efficiency after one single addition of the enzyme (90%) was improved by a threefold addition (0, 8, 12 h). Control incubations without enzyme contained <1% of the total volatiles extracted from parallel samples in presence of enzyme.

The buffer solution was extracted again four times as above to determine the portion of originally bound volatiles. Gas chromatographic analysis and quantification via internal standard were as above [20]. To exclude a possible isomerization of enantiomer menthols during the selectivity experiment the alcohols were esterified with methoxy-trifluorophenylacetic acid chloride and the diastereomers separated using an achiral (CW 20 M) stationary phase (170 °C isothermal) according to [21].

Analysis of the carbohydrate moieties was carried out using high performance silica gel sheets 60 F_{254} (Merck). The liquid phase was $CHCl_3$/MeOH/H_2O (16/9/2), and detection reagent was aniline/diphenylamine/H_3PO_4.

Determination of chlorophyll

The sum of chlorophyll *a* and *b* was determined according to Hüsemann *et al.* [22].

Results

Glycosilation by different cell cultures

Trace amounts of natural volatile compounds containing an alcoholic function such as phenylethanol occasionally appear in hydrophilic fractions of plant cell cultures after addition of hydrolytic enzymes. An exhaustive solvent extraction step prior to the addition of the enzyme ensures that these flavour compounds are liberated from bound forms.

Starting from this recurring observation high density suspension cultures of various species were prepared and incubated with a mixture of terpenoid and aromatic alcohols (Table I). While the rosemary and grape cell cultures stopped growing, the fresh weight of pear and peppermint cultures increased further. The total concentration of exogenous substrates was adjusted to a level similar to the concentrations of these alcohols in related essential oil-bearing economic plants (*ca.* 260 ppm, calc. from the average mol wt.).

The conversion of the single substrates proceeds rather non-specifically: linalool, a genuine constituent of muscat grape fruits, shows the lowest conversion rate in the muscat grape culture; menthol, not genuine in rosemary and pear, is well converted in both cell cultures. The highest conversion rates were found for linalool and phenylethanol in the peppermint culture. Unequivocal differences between heterotrophic and photomixotrophic cells cannot be derived from the figures of Table I.

Table I. Conversion of alcohols (%) to glycosides in various cell suspension cultures (mixture of alcohols, 0.3 μmol·g fr.wt.$^{-1}$ each, cell density 230 mg·ml^{-1}, incubation period 24 h, cell age 3 days).

Substrate	*Rosm. off.*	*Pyrus comm.*	*Vitis vin.*	*Mentha pip.*
	Photoheterotrophic cells		Photomixotrophic cells	
Linalool	9.4	15.4	0.6	58
Menthol (rac.)	14.5	29.2	3.4	48
Geraniol	3.3	5.2	1.5	14.1
Benzyl alcohol	14.1	12.5	19.2	44.3
Phenylethanol	23	32	9.6	70.4
Farnesol	29.6	14.5	17.3	14
Sum (μmol·100 g fr.wt.$^{-1}$)	43.2	14.4	14.8	49.8

Effects of substrate concentration

Suspension cultures of *Mentha,* incubated with exogenous menthol, were chosen for the following experiments for a number of reasons: the conversion rate is high (Table I), both the culture and the substrate are easy to handle, the culture was available in hetero- and photomixotrophic state, and reference data for the intact peppermint leaf were published [23].

A three days old culture of heterotrophic cells was equally divided into four flasks and incubated with different menthol concentrations. Although a formation of menthyl glycosides was obtained at all substrate concentrations tested (5–40 μmol·g fr.wt.$^{-1}$), menthol concentrations greater than 20 μmol·g fr.wt.$^{-1}$ provoked a discolouration of the culture and a stop in fresh weight increase. In the range between 5 to 10 μmol about one third to one fourth of the substrate is glycosidically bound under these conditions: cell density 35 mg·ml^{-1}, incubation period 24 h.

Time course of glycoside formation

Menthol was added to parallel cultures obtained from one stock suspension at the same point of the growth cycle, and incubated for different periods of time.

The glycosilation of menthol by peppermint cultures is a rapid reaction (Table II). Already after 12 h more than one third of the exogenous substrate occurs glycosidically bound. A peak concentration is reached after 24 h. This concentration exceeds the reported values of the glucoside in leaves of Japanese mint *(M. arvensis)* [23] by a factor of more than 20.

Table II. Incubation period and formation of menthyl glycosides in peppermint cultures (5 μmol menthol·g fr.wt.$^{-1}$, cell density 140 mg·ml^{-1}, cell age 4 days).

Incubation period [h]	12	24	36
Yield of glycosides (μmol·g fr.wt.$^{-1}$)	1.75	2.39	2.08
[%]	36.7	57.3	56.1

As indicated by the figures after 36 h of incubation the concentration of the glycosides decreases in the course of a prolonged cultivation. In four subsequent experiments the concentrations of glycosides were lower after 36 h as compared to parallel 24 h incubations. The data selected for Table II are representative, indicating a general tendency. With respect to the continuous increase in cell fresh weight this decrease of glycosides becomes even more evident from the fresh weight related figures in Table II.

Growth cycle and distribution of menthol

Parallel cultures with identical inocula were grown under the same conditions, and each flask supplied with a definite amount of menthol for a constant incubation period at different points of the growth cycle (Table III).

In contrast to the production of typical secondary metabolites in cell cultures, the glycosilation of menthol is most pronounced in early log-phase cultures. The absolute concentration of extracellular glycosides remains low during the growth cycle. A slight relative increase within the sum of glycosides towards the end of cultivation may be explained by

Table III. Glycosilation efficiency during the growth cycle and distribution of added menthol (%) in peppermint cultures (8.8 μmol menthol · g fr.wt.$^{-1}$ inoculated, incubation period 24 h).

Day of cultivation	0	4	6	7	10	13	16	19
Total fr.wt. [g]	10	11	16.5	18	22.5	32	50	49
Cellular glycosides	–	53.4	57.3	45.9	38.4	21	3.4	1.1
Glycosides in medium	–	1	1	0.3	–	0.2	0.2	0.1
Cellular menthol	–	0.4	–	0.1	0.1	0.2	0.4	18.2
Menthol in medium	–	2.8	–	<0.1	<0.1	<0.1	2.8	4.7

progressive cell lysis. The portion of free menthol in both cell saps and nutrient medium is similarly small and does not exceed 4% until the phase of cell death is reached.

A minimum of total menthol (<7%) is recovered from a culture which is just entering the stationary phase (day 16). The fate of the remaining menthol is presently unknown. Volatile transformation products, obtained on incubation of other essential oil constituents in peppermint cultures [24, 25], were detected neither in media nor in cell saps of the cultures.

Characterization of the glycosides

High performance thin layer chromatography (h.p.t.l.c.) was used to separate the components of glycoside containing fractions. While in control cultures, cultivated without added alcohols, only traces of glycosides were detectable, more than 10 components could be clearly distinguished in glycoside fractions isolated from cells after alcohol feeding. These components possess, based on the method of visualization, one or more carbohydrate moieties. The quantitatively dominating component shows a R_f-value in the range of monoglycosides, while the other spots with greater R_f-values point to the presence of isomer di- and oligosaccharide structures. The portion of oligosaccharides depends, *i.a.*, on the substrate concentration and the incubation conditions.

A carbohydrate-free enzyme preparation was used to detect the glycosidic carbohydrate moieties in extracts of hydrolysates. D-glucose is the only carbohydrate detectable in hydrolysates from cells grown in standard liquid media. The enzymic hydrolysis is less efficient when a purified β-glucosidase is used. This result indicates that non-β-glucosidic linkages may occur. However, a lower substrate specifity or an enhanced sensitivity of the β-glucosidase towards a natural inhibitor may contribute to the lower yields. On addition of other carbohydrates to the nutrient medium an increased diversity of products with yet unknown structures was observed by h.p.t.l.c.

Effect of cell aggregation

The particle sizes in peppermint suspension cultures comprise a wide range from single cells up to clusters with several 100 cells. While small aggregates facilitate substrate and product transport and should, therefore, show an accelerated primary metabolism, larger aggregates build-up gradients and intercellular communication across the symplast which in turn could promote biochemical differentiation [26].

A stock suspension culture was divided into fractions of different particle distribution by sieving. Parallel flasks, each containing one of the fractions were set to identical fresh weight contents and exposed to the same menthol concentration (Table IV). Obviously, the glucosilation of menthol is closely correlated with the particle size: the yields are constantly improved with decreasing cell aggregation.

Effect of light

Heterotrophic calli of peppermint were converted into photomixotrophic ones with an early stationary-phase content of *ca.* 50 μg chlorophyll · g fr.wt.$^{-1}$. A dark-green grey, fine suspension was obtained. Heterotrophic and mixotrophic cultures were grown

Table IV. Particle size distribution and glucoside formation in peppermint cultures (2.5 μmol menthol · g fr.wt.$^{-1}$, cell density 140 mg · ml^{-1}, incubation period 24 h, cell age 4 days).

Particle fraction	coarse	medium	fine
Aggregate size [μm]	>800	400–800	<400
Yield [μmol · g fr.wt.$^{-1}$]	0.44	0.71	1.05

in triplicate set-ups containing identical nutrient media and inocula.

Substrate-dependent differences of the glucosilation rates are found (Table V): menthol and two out of the four aromatic alcohols are significantly better converted by light-grown cultures. The glucosilation of linalool, benzyl alcohol, and phenylethanol is, within the analytical deviation, not affected by illumination.

Table V. Light and glucosilation efficiency (% of added substrate) in peppermint cultures, measured by using a mixture of alcohols (each alcohol 0.5 μmol·g fr.wt.$^{-1}$.

Substrate	Heterotrophic	Photomixotrophic
Menthol	21	37
Methyl salicylate	1.7	3.9
Eugenol	1.8	3.0

Substrate selectivity

Equal amounts of l- and d-menthol (7 μmol·g fr.wt.$^{-1}$) were added to peppermint cultures grown in duplicate set-ups under the same conditions (cell density 90 mg·ml^{-1}, incubation period 24 h, cell age 3 days).

Under these conditions, 35.6% conversion of d-menthol are opposed to 44.8% of l-menthol, indicating a slight preference for the natural enantiomer. The ratio of the conversion rates of the l- and d-forms is almost constant during an incubation interval of 48 h.

Discussion

Glycosidically bound volatile flavours are known from a number of *in vivo* plant cells, among them members of Lamiaceae (Peppermint leaves [23]), Rosaceae (rose petals [27], apple [28]), and Vitaceae (muscat grape [29]). A good part of our present knowledge of the involvement of glycosides in the pathways of volatile secondary constituents comes from work with peppermint leaves.

Although a rapid metabolic turnover of monoterpenes in mature peppermint leaves was early recognized [30], the participation of glycosides was demonstrated not before 1979 [31]. Studies with tritium labelled l-menthone and *M. piperita* showed that this key intermediate in flavour biogenesis is reduced to the epimeric alcohols l-menthol and d-neomenthol. While the resulting l-menthol and its acetylation product appear in the volatile fraction, the fate of d-neomenthol is different due to compartmentalized dehydrogenase isozymes: d-neomenthol is mainly converted to its β-D-glucoside, transported to the rhizome, and there degraded to acetyl-CoA and reduced pyridine nucleotide. The amount of menthyl glucoside formed is negligible compared to the neomenthyl glucoside [32, 33].

The high glycosilating activity of *in vitro* cultured cells of *M. piperita* for menthols and most of the alcohols tested (Table I) reflects a different metabolic situation. *In vitro* cells are forced to continuous elongation and division by exogenous phytohormones and may, thus, transform even foreign exogenous substrates to carbon and energy followed by a transfer to primary pathways. The use of glycosides may facilitate the transport to the catabolic enzymic sites. This view is supported by the low substrate specifity (Table I), by the constantly increasing respiration rates in the presence of increasing concentrations of exogenous terpenes, and by the sometimes rapid conversion of exogenous terpenoid substrates to better degradable compounds (*e.g.*, geraniol → geranic acid) [34, 35].

In vitro cells do not dispose of specialized storage structures to separate lipophilic compounds from sensitive membranes. An efficient glycosilating system with low K_m-values and low product inhibition, as indirectly indicated by Table II, is able to protect the cells from toxic effects exerted by an excess of lipophilic substrates. The maximum activity early in the growth cycle (Table III) suggests that glycosyl transferases which usually may synthesize and reconstruct cell wall polymers could be involved.

The enzymic background of light-stimulated glucosilation of menthol and some phenolics (Table V) is a matter of speculation as well, but the fact *per se* was observed for cardiac or flavonoid glycosides frequently [36]. As the monoterpene content of peppermint leaves is twice as much under high light intensity compared to low light [37], a concurrent stimulation of an accessory activity would be physiologically reasonable.

In conclusion, plant cell suspension cultures are well-suited to further study the catabolism and especially the glycosilation of volatile flavour compounds in plants. Moreover, the biotechnological production of glycosides using plant cell cultures could be sug-

gested. Glycosides are generally more stable and better water-soluble than the free aglyca and could be of interest to the pharmaceutical and food industry due to their physiological activities, *e.g.*, as bittering or flavouring agents [38].

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