Effects of Plant Bioregulators on the Production of Iridoid Derived Terpenoids in *Valeriana wallichii* and *Fedia cornucopiae* Cell Suspension Cultures

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Dedicated to Professor Erich Hecker on the occasion of his 60th birthday

Plant Bioregulators, Valepotriates, Suspension Cultures, *Valeriana wallichii, Fedia cornucopiae*

Four plant bioregulators were tested for their effects on production of valepotriates in *Valeriana wallichii* and *Fedia cornucopiae* cell suspension cultures. Concentrations of more than 10 ppm reduced valepotriate yield. At lower concentrations production was increased. For optimal activity, bioregulators had to be applied during early exponential growth, up to day 8 of the growth cycle. At equimolar concentrations dimethylmorpholinium bromide (4 ppm) and dimethylpiperidinium chloride (3 ppm) significantly improved total valepotriates in *V. wallichii* (up to 23%) and in *F. cornucopiae* (up to 50%). 2-(3,4-dichlorophenoxy)-triethylamine (6 ppm) and 2-(3,5-diisopropylphenoxy)-triethylamine (6.4 ppm) increased valepotriate production in both cell cultures up to 40%. With dimethylpiperidinium chloride and dimethylmorpholinium bromide the ratio of monoene to diene valepotriates in both cell systems was significantly shifted to the monoene compounds. A general use of these bioregulators to increase production of terpenoid secondary metabolites in plant tissue cultures is indicated.

Introduction

One of the major problems with secondary metabolite production in plant tissue culture is the low synthetic capacity compared to the whole plant [1]. Economically profitable productions are rare exceptions [2, 3]. Many methods have been applied to improve the yield of cell culture systems, such as optimization of culture medium and cultivation conditions [4], addition of a second phase to the medium to enable external accumulation of lipophilic products [5], or selection of high producing cell variants [6]. Many of these selected cell lines are biochemically very instable and often have to be reselected for their productivity [7]. However, treating cell cultures of *Valeriana wallichii* with colchicine, cell lines were selected which showed a permanent increase in secondary metabolite production (0.012% to 1.12%) [8]. Since treatment of other species didn't give similar results, there was still the necessity for a reliable method to improve the productivity of plant tissue cultures for a biotechnological application.

A suitable approach to increase the production of secondary metabolites of terpenoid origin was the use of plant bioregulators. Bioregulators were commonly used to alter the process of plant development [9]. Compounds such as 2-chloroethyl-trimethylammonium-chloride, CCC [10] or AMO-1618 [11] exert their effect by interfering with gibberellin biosynthesis.

Early work by Yokoyama and co-workers showed that 2-(4-chlorophenylthio)-triethylamine (CPTA),

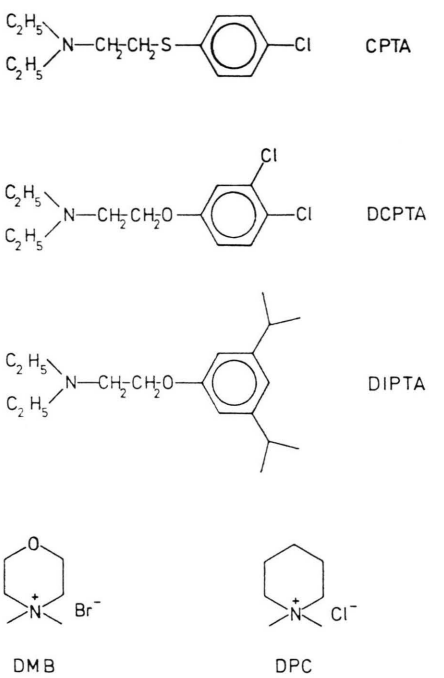

Fig. 1. Structures of applied bioregulators.

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in the basic structure related to CCC, induced carotenogenesis in citrus [12]. A large number of new derivatives has been synthesized and tested for their structure-activity relationship on the synthesis of carotenoids and polyisoprenoids [13]. The results implied that this class of compounds may be general inducers of terpenoid biosynthesis. Interesting for our work was the fact that the same compounds were able to improve the production of indole alkaloids in *Catharanthus roseus* cell cultures, with an iridoid part being of terpenoid origin [14].

Since the valepotriate skeleton is also of iridoid nature we found it promising to test plant bioregulators for their effect on production of valepotriates. Studies were conducted with suspension cultures of *Valeriana wallichii* and *Fedia cornucopiae*. For both cell systems, the cultivation conditions, growth and valepotriate production were well known. Two derivatives of CCC, dimethylmorpholinium bromide (DMB) and dimethylpiperidinium chloride (DPC) [15], and two inducers of carotenogenesis, 2-(3,4-dichlorophenoxy)-triethylamine (DCPTA) and 2-(3,5-diisopropylphenoxy)-triethylamine (DIPTA), were chosen as bioregulators.

Material and Methods

Cell cultures

The suspension culture of *Valeriana wallichii* was established in 1979, underwent a colchicine treatment and since yielded high amounts of valepotriates (1–1.5% of dry weight) [16]. The cell line produces a total of 10 diene and 2 monoene valepotriates as listed in Table I.

The suspension culture of *Fedia cornucopiae* was initiated in 1982 from a four year old callus culture [17] and had a valepotriate production of about 1.5% of dry weight. A colchicine treatment did not alter the valepotriate yield, but suppressed root differentiation [18]. The cell culture produced three diene valepotriates known from *V. wallichii* and two monoene valepotriates (Table I).

Table I. Structures of diene and monoene type valepotriates of *Valeriana wallichii* and *Fedia cornucopiae*.

Diene type Valepotriates	R^1	R^7	R^{11}	*V. wall.*	*F. corn.*	
Homovaltrate	(β-Me)-Val	iVal	Ac	+		
Isovaltrate	iVal	Ac	iVal	+	+	
Valtrate	iVal	iVal	Ac	+	+	
V (Homoisovaltr.)	(β-Me)-Val	Ac	iVal	+		
B (DIA-Valtrate)	iVal	Ac	Ac	+		
D (Homo-B)	(β-Me)-Val	Ac	Ac	+		
Acevaltrat	iVal	(β-OAc)-iVal	Ac	+		
Z	iVal	iVal	(β-OH)-iVal	+	+	
Y (Homo-Z)	iVal	(β-Me)-Val	(β-OH)-iVal	+		
F	structure not yet determined			+		
Monoene type valeopotriates	**R^1**	**R^7**	**R^{11}**	**R^5**		
Didrovaltrate	iVal	Ac	iVal	H	+	
Acetoxyhydroxy-didrovaltrate (AHD)	iVal	Ac	(α-OAc)-iVal	OH		+
Isovaleroxyhydroxy-didrovaltrate (IVHD)	iVal	Ac	(α-O-iVal)-iVal	OH		+
Hydroxy-didrovaltrate (M)	iVal	Ac	iVal	OH	+	

Ac = acetyl, Val = valeryl, iVal = isovaleryl.

Both cultures followed the same growth cycle with exponential growth from day 3 to 15. Valepotriate production increased parallel to growth.

Cultivation conditions

The cell cultures were maintained on a basic Murashige and Skoog nutrient medium containing 30 g/l sucrose and supplemented with 2 mg/l NAA and 1 mg/l kinetin. Subcultivation of 15 g of wet callus to 90 ml medium in 200 ml Erlenmeyer flasks was every 14 days. Cultivation was on a rotary shaker (Pilot-Shake, Braun-Melsungen) with 100 rpm in continuous light (2000 lux) at 25 °C.

Preparation of extracts

Freeze-dried callus material (1.0 g) was extracted twice with 100 ml CH_2Cl_2 for 10 min at 4 °C using an Ultra-Turrax homogenizer (Type 18/10, 20000 rpm, Janke & Kunkel). The extracts were filtered and evaporated to dryness under vacuum at max. 40 °C. The residue was dissolved in 3.0 ml methanol, kept for 12 h at −20 °C to precipitate all waxes and then filtered. The extract obtained was ready for direct HPLC analysis.

Valepotriate analysis

HPLC analysis was performed with two pumps, Waters 6000A with solvent programmer M 660, injector Waters U6K and a variable UV detector (Schöffel GM 770). A prepacked column Lichrospher RP 18, 5 µ, 4×250 mm (Merck) with a guard column was used. Water was double distilled and MeOH HPLC-grade (Baker). Solvent mixtures were filtered and degassed prior to use.

Separation of valepotriates was through linear gradient elution: Pump A 40% MeOH, Pump B 90% MeOH, initial cond. 75% B, final cond. 95% B, flow rate 1.2 ml/min, time 5 min, UV detection of diene valepotriates at 254 nm, of monoene valepotriates at 208 nm.

For quantitative analysis *n*-pentylbenzene was used as internal standard. Exact determination of respective factors is described elsewhere [19]. The peak areas were obtained through integrator, the valepotriate content in % dry weight and the relative amount of a compound compared to total yield was then calculated by a computer program.

Plant bioregulators

DMB and DPC were supplied by BASF Agricultural Research Centre, Limburgerhof. DCPTA and DIPTA were gifts from Prof. Henry Yokoyama, USDA, Pasadena, Ca.

Application of plant bioregulators

Aqueous solutions of plant bioregulators were added to the culture through a 0.22 µm millipore filter. Cultivation was up to day 14. The pH of the medium was determined and the cells were harvested. Fresh weight was recorded immediately, dry weight right after freeze-drying (2 days).

In preliminary experiments three cultures were examined. For statistical evaluation all other experimental conditions were determined fivefold.

Results

Determination of parameters for application of bioregulators

Various concentrations of DMB were added to cultures of *V. wallichii* and *F. cornucopiae*. Since valepotriate production and cell growth were associated [16], DMB was added on day 8, during the exponential growth. With 50, 100 and 200 ppm, total valepotriate yield was significantly reduced in both cell systems, 2 and 4 ppm, however, resulted in a 20% increase (Fig. 2a+b).

Cell growth and pH of the medium was not affected in *V. wallichii* even with high concentrations. Only with 200 ppm, growth of *F. cornucopiae* was

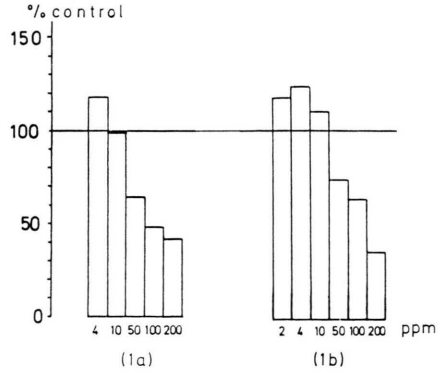

Fig. 2. Effect of various concentrations of bioregulator DMB on valepotriate production of cell cultures of *V. wallichii* (**1a**) and *F. cornucopiae* (**1b**) applied on day 8 of the growth cycle.

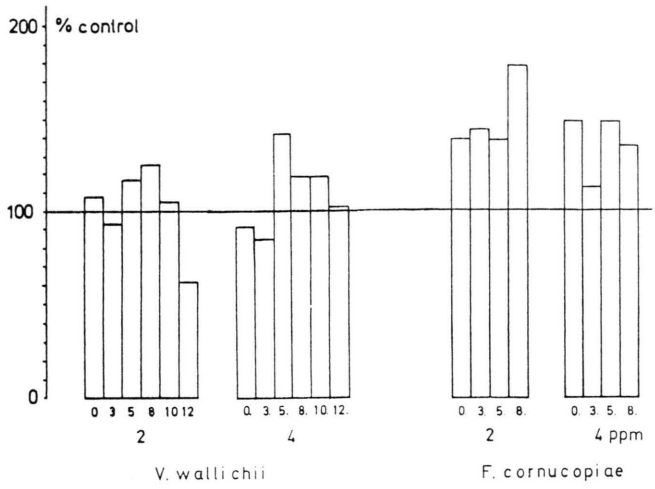

Fig. 3. Effect of 2 and 4 ppm of bioregulator DMB – when applied on different days of the growth cycle – on valepotriate production of *V. wallichii* and *F. cornucopiae*.

slightly reduced (20%). The pH varied between 5.4 and 5.7 and was not related to any DMB concentrations applied.

As for the optimal day of application, 2 and 4 ppm DMB were added at different days during the growth cycle. With *V. wallichii,* day 5 and 8, and with *F. cornucopiae,* day 3 to 8 gave the best results (Fig. 3).

From there we concluded that concentrations of DMB lower than 10 ppm added during early exponential growth improved valepotriate yield in both cell systems.

Effect of DMB and DPC on valepotriate production

The two structural analogues were applied at equimolar concentrations (0.02 mmol), 4 ppm DMB and 3 ppm DPC, to cultures of *V. wallichii* on day 5 and 8 and to *F. cornucopiae* on day 3, 5 and 8.

The total valepotriate yield of *V. wallichii* was significantly increased with DMB added on day 8 (15.3%) and with DPC added on day 5 (23.0%), see also Table II. DPC also improved, however not significantly, total valepotriates when applied on day 8 (20%).

Alterations of the valepotriate spectrum were observed. Data for valtrate, isovaltrate, B and Z and the two monoenes didrovaltrate and M (5-hydroxydidrovaltrate), counting for 76% of total valepotriates in controls, are represented in Fig. 4. Significant increase of total valepotriate concentration in *V. wallichii* was coherent with a stimulation of monoene valepotriates, up to 137% (M) and 185% (didrovaltrate) above controls. Hence improving monoene concentration from 11% to 23% of total yield.

In all experimental series, DMB and DPC improved valepotriate yield of *F. cornucopiae* (Table II). The effect of both bioregulators was strongest when applied on day 8 of the growth cycle, thus improving total valepotriates of 40% (DMB) and 50% (DPC) and rendering a content of more

Table II. Effect of equimolar concentrations of bioregulator DMB (4 ppm) and DPC (3 ppm) on total valepotriate yield of *V. wallichii* and *F. cornucopiae*. Bioregulators were added to the cell cultures on day 5 and 8 *(V. wall.)* or 3, 5 and 8 *(F. corn.)* of the growth cycle. Significant increase in production, absolute and in % of controls.

	Valepotriate yield % dry weight	Standard deviation	Increase in production abs.	%
V. wallichii				
controls	1.612	0.180	–	–
4-DMB-5	1.372	0.107	−0.240	−15.0
4-DMB-8	1.857	0.130	0.263	15.3*
3-DPC-5	1.983	0.195	0.317	23.0*
3-DPC-8	1.932	0.305	0.320	19.9
F. cornucopiae				
controls	1.502	0.096	–	–
4-DMB-3	1.684	0.178	0.182	11.4
4-DMB-5	1.742	0.093	0.240	15.9*
4-DMB-8	2.101	0.199	0.599	40.0*
3-DPC-3	1.745	0.070	0.243	16.0*
3-DPC-5	1.839	0.148	0.337	22.5*
3-DPC-8	2.258	0.299	0.756	50.3*

* Confidence interval 95%.

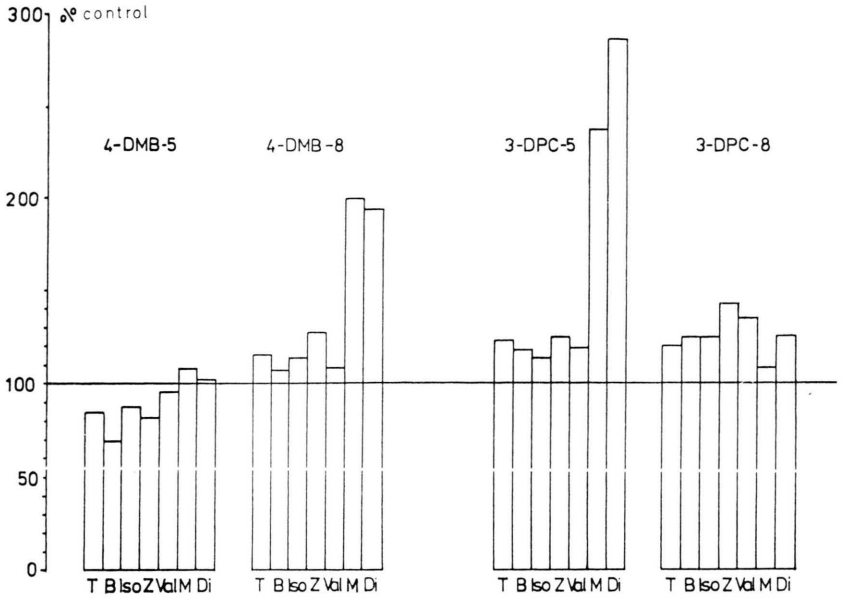

Fig. 4. Effect of bioregulator DMB (4 ppm) and DPC (3 ppm) on valepotriate production of *V. wallichii* added on day 5 and 8 of the growth cycle. Total valepotriate yield and concentrations of diene (valtrate, isovaltrate, B, Z) and monoene compounds (M and didrovaltrate) are shown in % of controls.

than 2% of dry weight, exceeding data reported for intact plants (0.62%) [17, 20].

Analysis of the two major constituents of *F. cornucopiae,* valtrate (71% of total yield of controls) and IVHD (20.3%), gave similar results as obtained for *V. wallichii.* In all series, concentration of the monoene type IVHD was significantly improved. Absolute and percent increase, exceeding the controls up to 90% and 130%, were always higher than for valtrate (Fig. 5 and 6), hence, increasing IVHD content up to 31% of the total valepotriates.

Effect of DCPTA and DIPTA on valepotriate production

Preliminary experiments showed that concentrations of DCPTA and DIPTA of more than 10 ppm reduced valepotriate production, lower amounts, however, increased the production in both cell culture systems. The main studies were then only conducted with *F. cornucopiae,* since the experiments with DMB and DPC have shown a better response to the bioregulator activity and the valepotriate spectrum was easier to control.

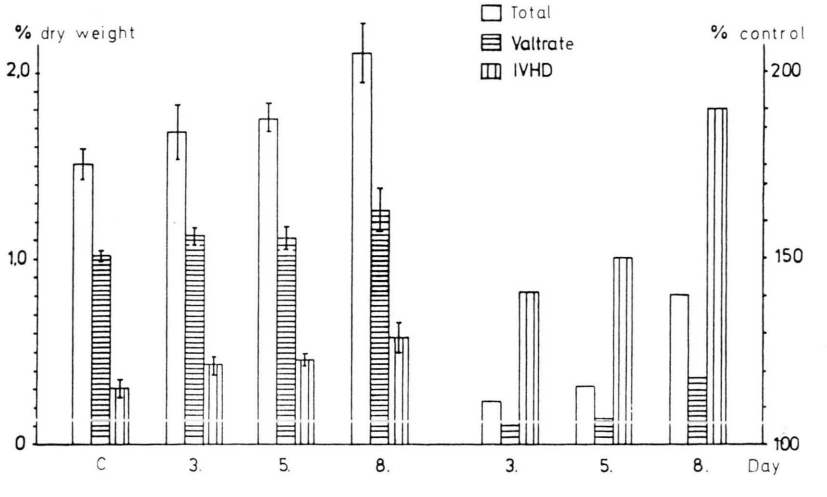

Fig. 5. Effect of bioregulator DMB (4 ppm, added on day 3, 5 and 8) on total yield of valepotriates, production of valtrate and IVHD in cell cultures of *F. cornucopiae.*

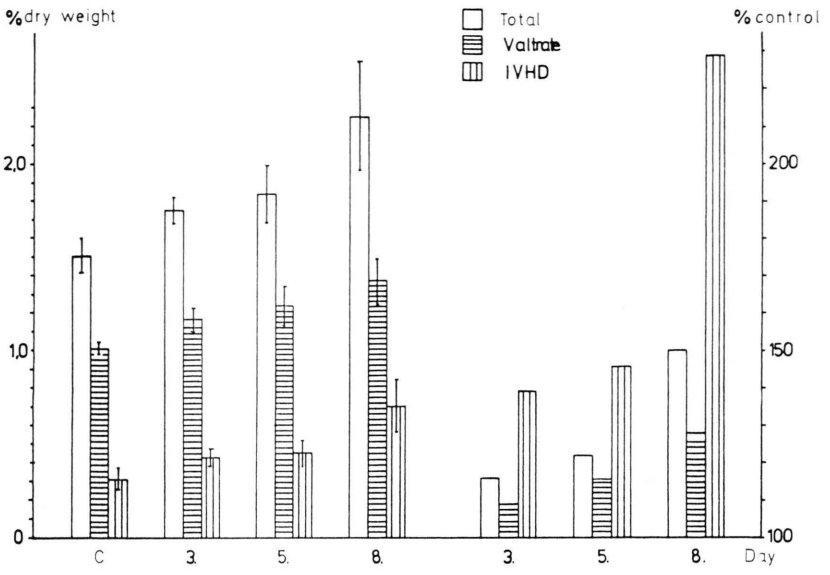

Fig. 6. Effect of bioregulator DPC (3 ppm, added on day 3, 5 and 8) on total yield of valepotriates, production of valtrate and IVHD in cell cultures of *F. cornucopiae*.

For comparison with DMB and DPC, both bioregulators were added at 0.02 mmol (6 ppm DCPTA, 6.4 ppm DIPTA) on day 8. Additionally 3, 9 and 12 ppm DCPTA were tested.

At equimolar concentrations, DCPTA and DIPTA significantly increased total valepotriate yield of about 40% (Table III), showing similar activity as DMB or DPC. With 3 ppm DCPTA, total

Table III. Effect of bioregulators DCPTA and DIPTA on total valepotriate production, valtrate and IVHD concentrations of *F. cornucopiae*, added on day 8 of the growth cycle. Significant increase in production.

		Yield % dw	rel %	Standard deviation	Increase abs.	%
	total	1.380	–	0.261	–	–
controls	Val	0.993	72.0	0.206	–	–
	IVHD	0.297	21.5	0.057	–	–
	total	2.348	–	0.449	0.968	70.1*
3-DCPTA-8	Val	1.625	69.3	0.306	0.632	63.6*
	IVHD	0.465	19.8	0.124	0.169	56.8*
	total	1.942	–	0.279	0.562	40.7*
6-DCPTA-8	Val	1.376	70.8	0.156	0.382	38.5*
	IVHD	1.390	20.1	0.071	0.093	31.3
	total	1.879	–	0.414	0.499	36.2
9-DCPTA-8	Val	1.335	70.1	0.267	0.342	34.5
	IVHD	0.378	20.1	0.081	0.082	27.5
	total	0.806	–	0.186	−0.574	−41.6
12-DCPTA-8	Val	0.583	72.4	0.133	−0.414	−41.3
	IVHD	0.170	21.1	0.042	−0.126	−42.6
	total	1.901	–	0.170	0.521	37.7*
6.4-DIPTA-8	Val	1.412	74.3	0.130	0.418	42.1*
	IVHD	0.311	16.3	0.522	0.014	4.8

* Confidence interval 95%.

valepotriates were also significantly improved (70%), with 9 ppm production was still increased, 12 ppm, however, rendered a lower yield (Table III), thus confirming the limitation for stimulatory activity to be 10 ppm.

The ratio diene to monoene constituents was not altered with DCPTA (or DIPTA). Stimulation of valtrate and IVHD was mostly comparable.

Discussion

The present investigation was to establish the use of plant bioregulators to improve secondary metabolism, *e.g.* valepotriate production, in plant tissue culture. With all four compounds, valepotriate yield could be significantly improved up to 23% in *V. wallichii* and up to 50% in *F. cornucopiae*. For optimal activity, the bioregulators had to be applied at concentrations of 0.01 to 0.02 mmol during early exponential growth, up to day 8 of the growth cycle. Our results are in accordance with findings by Yokoyama [13] and Lee [14] and thus indicating that these plant bioregulators in the future may generally be used to improve terpenoid secondary metabolite production in cell cultures. Gene derepression as a possible mode of action has already been postulated by Yokoyama [13]. Adding our results, this seems even more plausible since regulation will have to occur at an early step in terpenoid biosynthesis common to all of the different products, polyisoprenoids, carotenoids as well as valepotriates or alkaloids possessing an iridoid moiety.

Consequently, the system bioregulator-cell culture (*e.g.* as established for *F. cornucopiae*) might prove very useful in further elucidation of the regulatory mechanisms and enzymatic systems involved in early terpenoid biosynthesis.

Both groups of bioregulators showed similar effects on total valepotriate yield. Gene derepression might induce synthesis of a precursor of the valepotriate skeleton, which then is transformed into the monoene and diene type valepotriates. With DCPTA and DIPTA formation of monoene and diene compounds was improved in equal amounts. Whereas with DMB and DPC, production of monoene type valepotriates was far greater enhanced. These findings indicate that DMB and DPC, in addition to their effect as general inducers, might also affect valepotriate formation. Though biosynthesis of the iridoid valepotriate skeleton *via* 10-hydroxygeraniol has been investigated [21], no final conclusions could be drawn to explain the formation of monoene and diene valepotriates. The most probable biosynthetic sequence for a diene valepotriate, *e.g.* isovaltrate, would be *via* didrovaltrate and 5-hydroxy-didrovaltrate, both present in *V. wallichii*. A possible explanation for the high accumulation of monoene valepotriates with DMB and DPC could then be a partial inhibition of the reaction from hydroxy-monoene to diene valepotriates. Interaction of bioregulators at two sites was also known from carotenogenesis where CPTA was first found to inhibit cyclase(s) and preventing β-carotin formation [12]. This would indicate that diene valepotriates are the final step in the proposed sequence of valepotriate biosynthesis, but this should further be examined.

Through application of DMB and DPC, cell cultures of *V. wallichii* thus may become valuable sources of didrovaltrate, which due to its structure (cyclic dialdehyde) has recently been considered an ideal starter for drug-design [22].

With bioregulators stimulating the production of terpenoid natural products up to 50% and so far exceeding concentrations found in plants, plant cell cultures may – despite the high costs involved – become of industrial value.

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