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FORMATION OF MONO- AND DITERPENOIDS BY CULTURED CELLS OF *THUJA OCCIDENTALIS*

JOCHEN BERLIN and LUDGER WITTE

GBF-Gesellschaft für Biotechnologische Forschung m.b.H., Mascheroder Weg 1, D-3300 Braunschweig, F.R.G.

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Key Word Index—*Thuja occidentalis*; cell cultures; monoterpenes; diterpenes; two phase culture.

Abstract—Cultured cells of *Thuja occidentalis* produce a variety of mono- and diterpenoids, the former of which are excreted into the culture medium. The yields of monoterpenes and a thujaplicinato-Fe(III)-complex in the medium were not proportional to the biomass levels. The reason for this observation is probably related to the mechanism of product release which may include mechanical removal of monoterpene containing cells from the cell aggregates. The effects of some culture conditions, eg. the use of various lipophilic traps, on growth and the formation of the terpenoids by the cultures is described.

INTRODUCTION

The formation of distinct levels of monoterpenes by cultured plant cells has only been demonstrated for a few plant species [1]. Among these cultures, those of *Thuja occidentalis* are unique as they excrete all their regular monoterpenes into the culture medium [2, 3]. We have maintained these cells on liquid B5-medium [4] for more than six years and have not noted significant changes in growth and productivity during this time. The cultures have remained highly aggregated (mean aggregate size: 20 mg fresh mass) and slow growing. The complete spectrum of mono- and diterpenes produced by the *Thuja* cultures has been described [2]. Three groups of terpenoids of *Thuja* cultures may be distinguished by their different accumulation behaviour. The regular monoterpenes (eg. terpinolene, terpinen-4-ol, 2-methyoxy-*p*-cymen-8-ol) accumulate in the culture medium, the diterpenes (eg. dehydroferruginol) and the tropolones (eg. thujaplicines) within the cells, while the thujaplicinato-Fe(III)-complex is found in both. For trapping the complete spectrum of excreted monoterpenes it was necessary to apply a two phase culture system [3]. The different places of accumulation require different strategies for exploiting metabolite production. As the continuous production of regular monoterpenes by a culture maintained for several years in liquid medium, is still a rather rare event, the main emphasis of our investigations was devoted to this group of metabolites. Here we wish to summarize our results for the two phase cultivation of *Thuja* cells with respect to growth and production of terpenoids.

RESULTS AND DISCUSSION

The basic growth and production characteristics of Thuja cells

Usually cell cultures are grown on the same medium until the growth kinetics indicate the beginning of the stationary phase. The behaviour of *Thuja* cultures under such conditions is shown in Fig. 1a. After 40 days of cultivation of biomass had increased 2.5–3 fold. The main constituents revealed by GC-analyses of cell extracts were the thujaplicines (α, β, γ) accounting for *ca* 0.2% of dry mass (Fig. 1b). Dehydroferruginol was the main diterpene with 0.02% of dry mass. The main regular monoterpene in CH_2Cl_2-extracts of the medium was 2-methoxy-*p*-cymen-8-ol. Up to 8 mg/l accumulated at cell densities of *ca* 80 g fresh mass/l (Fig. 1c). The medium of shake cultures was reddish coloured due to the presence of the thujaplicinato-Fe(III)-complex. Without shaking, the red complex precipitated slowly from the medium together with cell debris. A part of the red colour was extractable into CH_2Cl_2. The accumulation pattern of the complex (Fig. 1c) indicated that the complex was modified or bound to other components in such way that it was no longer extractable.

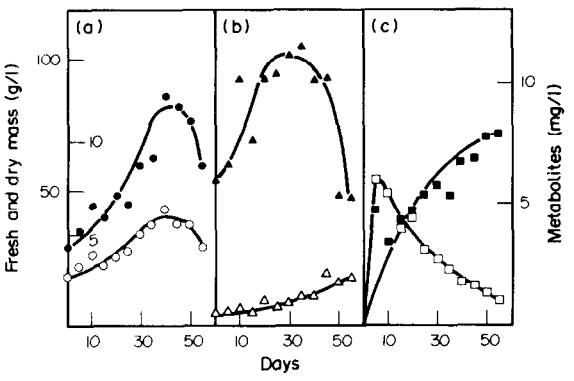

Fig. 1. Growth and production kinetics of *Thuja occidentalis* cell cultures. a: Fresh mass (●), dry mass (○); b: α, β, γ-thujaplicines (▲), dehydroferruginol (△) in the CH_2Cl_2-cell extracts; c: 2-methoxy-*p*-cymen-8-ol (■), thujaplicinato-Fe(III)-complex (□) in the CH_2Cl_2-extracts of the culture medium.

Comparison of the basic growth and production characteristics with two-phase culture systems

The basic kinetics of Fig. 1 show that under these conditions the higher lipophilic compounds released into the medium were not quantitatively trapped and that the complex released was not readily quantifiable. Thus the cells were also grown in the presence of lipophilic traps (Fig. 2). Growth was a little retarded in the presence of hexadecane, while the presence of XAD-2 had a slightly stimulatory effect.

The beneficial effect of XAD on growth during the first stages of the cultivation period was reproduced in several independent experiments. However, the final biomasses of one- or two-phase cultures were similar (Figs 1 and 2). In the presence of hexadecane (Fig. 2a), the main regular monoterpene was terpinolene followed by terpinen-4-ol. Terpinolene was never found without trapping. Terpinen-4-ol levels were distinctly increased in the presence of hexadecane and surpassed 2-methoxy-*p*-cymen-8-ol levels by 25–40%. As extraction of monoterpenes from liquid traps may cause some technical problems, we also tried to trap the monoterpenes on solid phases such as XAD and RP-8. The results were disappointing, however, as the monoterpene levels were rather low. Terpinolene was only detected sporadically. While hexadecane had absorbed 8 mg terpinen-4-ol/l after 20 days (Fig. 2a), only 2 mg/l (data not shown) were found for XAD-2 and RP-8. As these compounds are known to absorb lipophilic monoterpenes, the low absorption was probably due to insufficient contact of the volatile compounds with the solid phases.

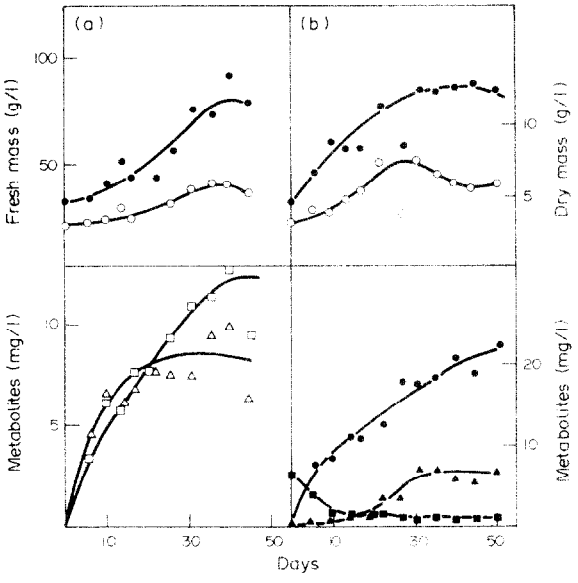

Fig. 2. Growth and production kinetics of *Thuja occidentalis* cell cultures grown in the presence of A = hexadecane (10/70 ml medium) or B = Amberlite XAD-2 fine. Only the production kinetics of those compounds are given which differ markedly from the one phase cultures of Fig. 1. a: Terpinolene (○), terpinen-4-ol (△) accumulated in the hexadecane phase; b: Thujaplicinato-Fe(III)-complex absorbed by Amberlite XAD-2 (●), thujaplicines (■) and dehydroferruginol (▲) in CH_2Cl_2-cell extracts

The levels of extractable thujaplicinato-Fe-complex were initially doubled in the presence of hexadecane (data not shown) [3] but levels declined in a way similar to that shown in Fig. 1. There was incomplete extraction of the iron complex from the medium as this remained reddish in the presence of hexadecane. However, in the presence of XAD or RP the medium was white, as all iron complex was absorbed by the traps. The levels of the iron complex accumulated in hexadecane reached extremely low levels at the end of the culture period, while it increased continuously in XAD (Fig. 2b).

The accumulation patterns of diterpenes and thujaplicines were not distinctly effected by hexadecane. In the presence of XAD, however, the thujaplicines decreased substantially (Fig. 2b) when compared to the accumulation curve of one-phase cultures (Fig. 1b). This may be due to the increased formation and release of the thujaplicinato-Fe-complex (see also Table 1). On the other hand, dehydroferruginol levels were increased 3–4-fold in the presence of XAD. Though the specific yields of dehydroferruginol were increased from 0.02–0.03% to nearly 0.1% it is evident that *Thuja* cultures are at present not competitive as producers of diterpenes. However, it is worthwhile noting that *Thuja* cultures accumulate the same or similar diterpenes as found in various other culture systems. Diterpenes of the abietane type such as ferruginol or hinokiol are evidently spontaneously expressed in many cell culture systems [5–9]. However, only the cultures of *Salvia miltiorrhiza* produced rather high levels of ferruginol [9]. A selected strain had a specific ferruginol yield of 13.7% of dry mass under special culture conditions and produced 730 mg/l within 16 days. The much lower specific product levels of *Thuja* cultures and the poor growth rates prevent their use for production of compounds stored within the cells.

Culture conditions for products released into the medium. As the most interesting compounds accumulated in the medium the usual growth technique was altered for *Thuja* cultures. It was noted that regular changes of the medium and the lipophilic trap every 7–10 days gave best yields of monoterpenes [3]. The question was, however, whether the continuous presence of a lipophilic phase would effect biomass production and monoterpene formation. We used mainly hexadecane as lipophilic trap as it proved to be non-toxic and was more convenient for GC-analyses of extracts than miglyol used earlier [3]. For monitoring growth the cell aggregates were transferred by a sieve spoon to fresh medium with or without hexadecane each week. (For maintenance of the cultures the old medium was decanted and replaced by fresh medium until the biomass for dilution was reached). Biomasses achieved after 4 passages starting from different initial sizes of inoculum are given in Table 2. The biomass increase was linear with time. As a first impression the growth rates do not seem to be distinctly increased by the weekly change of the medium when compared with the growth curve of Fig. 1. In many independent experiments it was noted that growth rates were higher at lower initial inocula as shown in Table 2. When the growth rate shown in Fig. 1 (initial inoculum 2 g fresh mass) was compared, however, with the growth rates of the cultures inoculated with 2.5 g (Table 2) it is clearly seen that the repeated exchange of the medium had a beneficial effect on growth. On the other hand, the growth retarding effect of hexadecane shown in Fig. 1b was even more pronounced by the repeated

Table 1. Distribution of the thujaplicinato-Fe(III)-complex between medium and cells when grown for one week in the presence of 10 ml hexadecane or 3.5 g Amberlite XAD-2

	Thujaplicinato-Fe(III)-complex (mg/flask)	
	Hexadecane	Amberlite XAD-2
Medium		
Lipophilic phase	0.8	2.5
Water phase	0.5	0
Cells	5.5	3.2
Total	6.8	5.7

The cultures were not diluted for this experiment and had final biomasses of 15.9 g (hexadecane) and 16.8 g (Amberlite XAD-2). The water phase were extracted with CH_2Cl_2.

Table 2. Effects of inoculum size on growth and monoterpene accumulation in the absence or presence of hexadecane as lipophilic trap

Inoculum (g)	Fresh mass (g)		Total yields (mg/flask)
Minus hexadecane			2-Methoxy-*p*-cymen-8-ol
2.5	10.2		0.60
5.0	16.0		0.61
7.5	15.8		0.60
10.0	17.0		0.55
Plus hexadecane		Growth factor	Terpinolene
2.5	6.4	(4.0)	1.68
5.0	10.2	(2.9)	1.12
7.5	10.6	(2.5)	1.36
10.0	10.8	(1.9)	1.32

The medium and the trap were replaced each week. The product levels are given as the total collected by the lipophilic trap or extracted from the medium by CH_2Cl_2 after four passages. The four-week growth factors of cells, previously maintained for four passages in the presence of hexadecane, when transferred back to medium without the lipophilic trap are given in parentheses.

change of medium and trap. Thus the continuous presence of hexadecane reduced growth of the cells greatly. However, when hexadecane treated cells were further cultivated without hexadecane original growth rates resumed immediately (Table 2). This was also shown for cells grown for even longer periods in the presence of hexadecane. Thus *Thuja* cells tolerate hexadecane at least for periods of 2–3 months without loosing viability. At cell densities of 16–18 g fresh mass/70 ml (corresponding to *ca* 250 g/l) biomass increase ceased even in the presence of fresh medium.

In Table 2 the product yields are given as the total of the four samplings. As expected the production rate, eg. of terpinolene, was distinctly increased by this culture technique (Fig. 2a, Table 2). However, according to Table 2, one may speculate that there was hardly any correlation between biomass and product formation. The flasks starting from 2.5 g inoculum, always having lower biomass present during the four passages, produced more terpinolene than cultures starting at 7.5 g initial inoculum. This problem was also seen when the weekly production rates of a single flask were followed. For example, the first sampling provided 42%, the second 17%, the third 10%, and the fourth 31% of the total monoterpenes collected after four passages. The initial biomass of one flask produced the same or even higher amounts of monoterpenes than the three-fold increased biomass of the fourth passage. The differences between the weekly collection seemed to vary depending on the density/age of the preculture which was diluted. In the absence of a lipophilic trap such observations may be explained by the fact that the volatile compounds are lost or are incompletely trapped in the water phase. The total absence of terpinolene in one-phase cultures is a justification for this assumption. In the presence of lipophilic traps the absence of a correlation may be explained by some negative effects of the chemical agent. A lack of correlation between biomass and production levels of monoterpenes could also be due to initial inocula with different productivities. This danger is undoubtedly greater with very slowly growing aggregated cultures such as *Thuja*. We have tried to analyse this problem by measuring repeatedly weekly production rates of parallel cultures with the same and different initial inocula.

In Table 3 the weekly production rates of six flasks with the same inoculum derived from the same precultures are given. The yields of the first week indicated rather small differences between parallels. The differences between the flasks increased after the second sampling. Generally the monoterpene yields of the second sampling were lower. Usually highest specific accumulation was found with cell cultures that had just been diluted. In all our experiments the weekly production rates of the same culture varied greatly (Table 3). This excludes the possibliity that the main reason for the apparently biomass unrelated production rates are due to cultures with different initial productivities.

In another experiment the productivities of parallel cultures with different initial inocula from the same preculture were tested (Table 4). Though the impact of the biomass for the production is clearly indicated, a close correlation of biomass and product yields was again not seen. The rather high product levels in Table 4 were probably due to the fact that the preculture had been maintained for two passages at rather high cell densities before dilution. Evidently it gives optimal cell densities for highest specific monoterpene release. At higher cell densities (15–18 g fresh mass/70 ml) the production of monoterpenes and of the thujaplicinato-Fe-complex were comparatively low despite fresh medium. When these cultures were diluted a burst of product release was seen. It may be postulated that this increased release is related to the mechanism of excretion of the products. In this context it may be worthwhile mentioning one experiment where we found only traces of monoterpenes and the iron complex in the medium.

Cells releasing their products to the culture medium may be maintained in aerated columns. Thus we compared growth and monoterpene production in a column reactor packed with 35 g cells and 50 ml medium plus hexadecane with the results obtained for a shake flask with the same inoculum. In both systems neither growth nor monoterpene production was observed. Similar observations were made following monoterpene production of *Thuja* cells in a column reactor, where the medium passed a XAD-bed [10]. In our experiment the hexadecane phases remained nearly colourless. But both cells from the column reactor and the shake flasks, immediately resumed monoterpene and iron complex release after the cells were diluted to 7.5 g/70 ml. We assume that for production and product release a certain mechanical stress is required so that the cells containing monoterpenes and the iron complex are removed from the cell aggregates. This is in accordance with the fact that cell debris, but not intact cells, are found in the culture medium and that the complex is associated and precipitated with this debris. At lower cell densities the movement of the aggregates in the flasks are greater and relatively more products are released. At higher cell densities this removal of monoterpene producing cells is hampered and potential releasing cells remain in the aggregates. When this culture is diluted, however, a relatively higher product release is noted during the first cycle.

Table 3. Weekly production rates of *Thuja* cells grown in the presence and absence of hexadecane

Culture	Terpinolene		Terpinen-4-ol		2-Methoxy-*p*-cymen-8-ol	
Week:	1	2	1	2	1	2
			(mg/flask week)			
Plus hexadecane						
A	1.12	0.54	0.42	0.30	0.20	0.20
B	0.98	0.80	0.26	0.21	0.17	0.16
C	0.90	1.17	0.31	0.42	0.14	0.21
Without hexadecane						
A			0.16	0.14	0.39	0.42
B			0.14	0.21	0.30	0.36
C			0.21	0.10	0.29	0.27

Initial inoculum with cells from the same batch 5 g/70 ml medium plus 10 ml hexadecane. Final biomasses after 2 weeks 8.6 ± 0.2 g.

Table 4. Comparison of terpinolene accumulation of *Thuja* cultures at various initial inocula

Initial inoculum (g)	Terpinolene (mg/flask)		
	Week 1	Week 2	Week 3
1.0	0.70/0.74		
2.0	2.65/1.82		
5.0	4.83/6.20	1.86/1.53	0.98/0.87
10.0	3.93/3.16	1.65/1.52	

The two precultures had a final biomass of 18 g/70 ml and were combined before dilution. The hexadecane phase was measured weekly.

We consider the proposed mechanism of release as the main reason for the biomass unrelated production behaviour of *Thuja* cells. However, the various aggregates of a culture may also have different production and growth phases which are difficult to distinguish due to the very slow growth. Producing, but non-growing aggregates, and growing, but poorly producing lumps, may exist and this ratio may change during the cultivation period. This may also explain the rather large differences in product yields as shown in Tables 3 and 4. The growth kinetics did not show the typical phases such as log, linear or stationary phase and thus production of monoterpenes could not be related to any special growth phase. At present it must be clearly stated that the monoterpene production behaviour of *Thuja* cells is not comparable with production kinetics found with rapidly growing cells where productivity can be related to biomass or culture phases.

It was observed that the lipophilic phase should be as thin as possible. Thus 5 ml hexadecane layered over 70 ml medium in a 200 ml Erlenmeyer flask absorbed the same amounts of monoterpenes and iron complex as a 10 ml layer. With thicker hexadecane layers accumulation may be impaired, as we found decreased yields of monoterpenes in the presence of 20 ml hexadecane. Cells grew slightly better in the presence of 5 or 10 ml hexadecane than of 20 ml. It has been reported that light may effect monoterpene formation in cultured cells [11, 12]. However, in the case of *Thuja* cultures growth rates and monoterpene accumulation in the hexadecane phase were similar in the light (16 hr-day) or in the dark.

As mentioned above the accumulation of the released monoterpenes on solid phases added to the culture medium was also tried. A great advantage of solid phases would be the possibility of extracting the monoterpenes from these materials into small volumes of the most convenient organic solvent. Various XADs and RP-8 were used. However, in shake cultures the absorption of monoterpenes by the solid phases could not compete with the liquid phases. According to our results we suggest harvesting the monoterpenes of *Thuja* cultures by trapping them during the first passage after dilution, and perhaps increasing the shaking speed during this time. To avoid the growth inhibitory effect of hexadecane and to avoid handling of hexadecane samplings with rather low monoterpene yields, the culture should then be allowed to grow up to higher cell densities without hexadecane or should be treated at intervals with hexadecane.

Accumulation of the thujaplicinato-Fe(III)-complex in the medium

When cells of *Thuja* cultures are extracted with organic solvents, the extracts are red due to the presence of the thujaplicinato-Fe(III)-complex. Though the cells contain more of the iron-complex than the medium, it may not be wise to sacrifice too many cells for recovery of this product as the growth rates of *Thuja* cells are poor. As shown in Table 1 hexadecane absorbed only 60–70% of the extractable portion of the complex. As culture time increased more and more of the complex could not be extracted with the liquid–liquid two phase cultivation and afterwards could not be extracted into methylene chloride from the water phase. When hexadecane and miglyol were compared for monoterpene absorption no differences were found. However, miglyol absorbed generally 20–30% more of the complex than hexadecane. Nevertheless, the water phase also remained red in the presence of miglyol. Thus the solid phases are undoubtedly the best for harvesting the iron-complex from the medium (Fig. 2, Table 1). When cells were grown for three consecutive cycles in the presence of Amberlite XAD-2 the many cell aggregates became grey and the cell extracts were nearly colourless. Thus phases of recovery would be required if the complex is collected on Amberlite XAD-2. Growth was not altered by the depletion of the iron-complex from the cells. As with the monoterpenes the levels of complex found in the medium were not proportional to the biomass in the flasks. The passage after dilution usually gave the highest specific yield and often total yields per week. At higher dilutions relatively more complex was found in the medium. Thus there is sufficient evidence that the monoterpenes and the iron complex are released from the aggregates by the same mechanism.

The monomer thujaplicines and the complexed thujaplicines in the cells account for more than 0.5% of the biomass. As additional iron-complex is found in the medium it is evident that thujaplicine formation seems to be the most active branch of terpenoid production in *Thuja* cells. Thus the *Thuja* cultures may not only be interesting as producers of monoterpenes but may also be useful for biochemical investigations of tropolone biosynthesis. Biochemical and microscopical studies may also reveal why the monomer thujaplicines are not released into the culture medium and which type of cell releases the iron complex and the monoterpenes.

EXPERIMENTAL

Cell culture. The initiation and maintenance of the *Thuja* cell culture has been described [2]. The initial inoculum for maintenance cultures was 7.5 g fresh mass/70 ml B5-medium. The medium was changed weekly and the cells were diluted when the biomass of one flask had reached 15–18 g. In the case of two phase cultures the lipohilic phases (hexadecane, miglyol [3], the various Amberlite XADs and RP-8 suspended in H_2O) were autoclaved before addition to the culture. Amberlite XADs were additionally purified by extraction with CH_2Cl_2. For comparative parallel experiments the biomass of various preculture flasks were collected on Buchner funnels and introduced on a sieve spoon into the flasks.

Analytical measurements. The characterization of the product spectrum and the conditions of capillary gas chromatography have been described recently [2, 3]. Quantification was performed by the use of calibration curves of authentic terpinolene and thymol for regular monoterpenes, of γ-thujaplicine, and methyl stearate for diterpenes. The calibration curves were not linear, as at lower concentrations relatively higher losses were noted. The thujaplicinato-Fe(III)-complex was measured in hexadecane, miglyol or CH_2Cl_2 at 540 nm.

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