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DETERMINATION AND QUANTIFICATION OF MONOTERPENOIDS SECRETED INTO THE MEDIUM OF CELL CULTURES OF *THUJA OCCIDENTALIS*

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Key Word Index—*Thuja occidentalis*; Cupressaceae; cell cultures; monoterpene accumulation; Miglyol.

Abstract—Cell cultures of *Thuja occidentalis* excrete monoterpenoids into the culture medium. Highly volatile monoterpene hydrocarbons were only detectable in the presence of Miglyol, a water-insoluble non-toxic trap for lipophilic compounds. In the presence of Miglyol the levels of oxygenated monoterpenoids were two-fold higher than in dichloromethane-extracts of culture media. The cells excrete up to 3 mg regular monoterpenoids/g dry wt/day. Continuous extraction of lipophilic compounds by Miglyol also allowed quantification of a Tris–thujaplicinato–(Fe III)-complex.

INTRODUCTION

We have recently shown that cell cultures of *Thuja occidentalis* L. produce oxygenated monoterpenes of the menthane type and tropolonate derivatives [1]. The most interesting observation was that these compounds were not accumulated in the cells but were released into the culture medium. For more than two years the culture medium has been replaced every 7–10 days and used as a source for the monoterpenoids. An easy way to collect excreted volatile compounds quantitatively is to add the water-insoluble non-toxic triglyceride Miglyol to shake cultures [2, 3]. In the present report we demonstrate that Miglyol was not only useful for the quantification of the excreted monoterpenoids but also helped to detect additional, very volatile monoterpene hydrocarbons which cannot accumulate in water.

RESULTS AND DISCUSSION

The monoterpenoid producing *Thuja* cultures grow rather slowly as small morphologically undifferentiated lumps on B5-medium [4] supplemented with 5×10^{-6} M 2,4-D. The cells were diluted every three months 1:1, while the culture medium was normally replaced every 7–10 days. When a capillary GLC-chromatogram of the dichloromethane-extract of the culture medium and the Miglyol phase of cultures grown in the presence of Miglyol were compared it was evident that the lipophilic agent had captured additional more volatile compounds (Fig. 1). These were identified by their mass spectra and retention indices as α-pinene, β-pinene, myrcene, limonene and terpinolene. The monoterpene hydrocarbons were transferred from Miglyol into a trap of liquid nitrogen with a stream of nitrogen. In this way 95% separation from the oxygenated monoterpenes was achieved. The structures of the two main compounds of monoterpene hydrocarbons were confirmed from their ^{1}H NMR spectra. The very volatile character of α-pinene was demonstrated when only 3% of added α-pinene was recovered from a culture medium kept for six hours on a shaker. If a culture releases volatile compounds into a culture medium a complete spectrum of the excreted compounds is only possible by the use of traps. For analytical purposes Miglyol is the easiest system to handle. Beiderbeck and co-workers have used this system with success for their crown-gall and habituated *Matricaria chamomilla* cell lines [2, 3]. Since the *Thuja* line requires 2,4-D the distribution of labelled 2,4-D between Miglyol and the culture medium was determined. After five days on B5, when 70% of 2,4-D was absorbed by the cells, 95% of the radioactivity remaining in the media was in the water phase, while only 5% was found in Miglyol. We have also tested other 2,4-D dependent cultures for growth inhibitory effects of Miglyol. A tobacco cell line was kept for six growth cycles in the presence of Miglyol without significant growth retardation. Thus, this method can be used as a general method for the trapping of volatile secondary metabolites excreted by cell suspension cultures.

The GLC-chromatograms (Fig. 1) show the qualitative differences of the monoterpene spectra of Miglyol treated and untreated cells. All compounds less volatile than terpinolene are present in both the Miglyol and the dichloromethane-extracts. A quantitative comparison (Fig. 2) showed that in the presence of Miglyol the yields of isolated oxygenated monoterpenes were doubled. It is assumed that the increase of products was not caused by increased synthesis but by the better solubility of volatile compounds in Miglyol. The main product, however, is the monoterpene hydrocarbon terpinolene. Up to 6.0 mg terpinolene were detected per flask during the first seven days of culture (Fig. 2). Thus, in the presence of Miglyol a synthesis rate of 3 mg monoterpenoids/g dry wt/day was calculated when the medium was changed every seven days. According to the dichloromethane-extracts only 0.8 mg/g dry wt/day was formed. The accumulation curve of terpinolene (Fig. 2) however, indicates that even in the presence of Miglyol a portion of the very volatile compounds was lost.

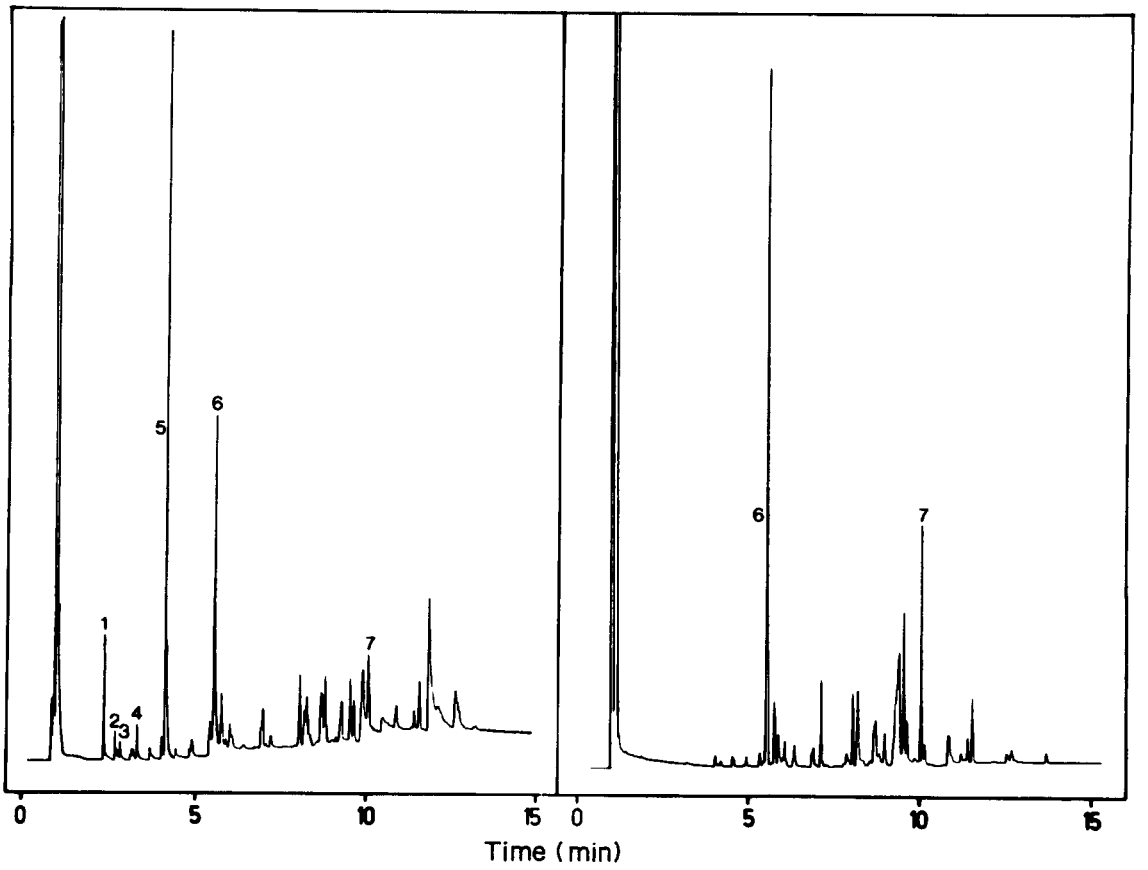

Fig. 1. Comparison of GLC-chromatograms of the Miglyol phase (left) and a CH_2Cl_2-extract (right) of the medium of *T. occidentalis* cell cultures. 1, α-pinene; 2, β-pinene; 3, myrcene; 4, limonene; 5, terpinolene, 6, terpinen-4-ol; 7, methoxy-*p*-cymen-8-ol. Most of the unnumbered peaks have been characterized recently [1]

Another advantage of using Miglyol for continuous extraction of lipophilic compounds is evident from the quantitative determination of the Tris–thujaplicinato–(Fe III)-complexes with and without Miglyol present (Fig. 3). The dichloromethane-extract of the culture medium and the Miglyol phase of *Thuja* are deep red in colour due to the presence of iron–tropolonate complexes [1]. These complexes become less extractable by the solvent during the culture period with a film developing between the two phases. Even extraction with dichloromethane–methanol or ethyl acetate–methanol did not give higher yields. Extraction with Miglyol gave the poorest results. We assume that this complex reacts with some other compound accumulating in the medium during the culture period. The continuous extraction of the iron complexes into Miglyol prevented this 'aggregation'. The accumulation pattern of the complexes in the presence of Miglyol (Fig. 3) agreed well with those of the regular monoterpenoids.

We have kept the cultures on various culture media to improve growth or to alter the monoterpenoid spectrum. The slow growth rate suggested that B5-medium was too rich. Indeed only 50% of sucrose was taken up when 5 g cells/70 ml were cultivated for 10 days. The remainder was cleaved by invertase in the medium to glucose and fructose. Even when the sugar source was reduced from 2 to 1%, 37% of the sugar still remained in the medium. The amount of phosphate taken up also depended on the concentration in the medium. From a solution of 0.125 mM and 1.25 mM potassium dihydrogen phosphate approximately 80% of these amounts were taken up. Since growth was not distinctly influenced by keeping the cultures on rich or poor media, the surplus is stored in the cells. It is known from other culture studies that the accumulated phosphate influences the expression of secondary pathways [5]. Cultures kept for three cycles on 1/10 B5 medium + 1% sucrose produced slightly higher levels (30%) of monoterpene hydrocarbons, while the level of oxygenated monoterpenoids was the same on B5 and 1/10 B5. The Miglyol phase of cultures grown on 1/10 B5 was rather colourless, as only one-fifth of the tropolonate complexes were formed due to the lack of iron. Experiments to alter the productivity of slowly growing cell cultures by changing the medium composition are often rather difficult to interpret. However, our data show that *Thuja* cultures react to such changes and therefore some improvements in manipulating the monoterpenoid formation is to be expected.

Monoterpenoids are rarely found in cell cultures. It is assumed that the lack of specialized storage cells accounts for this. With the *Thuja* cell cultures we have established a system in which the culture medium is the storage site.

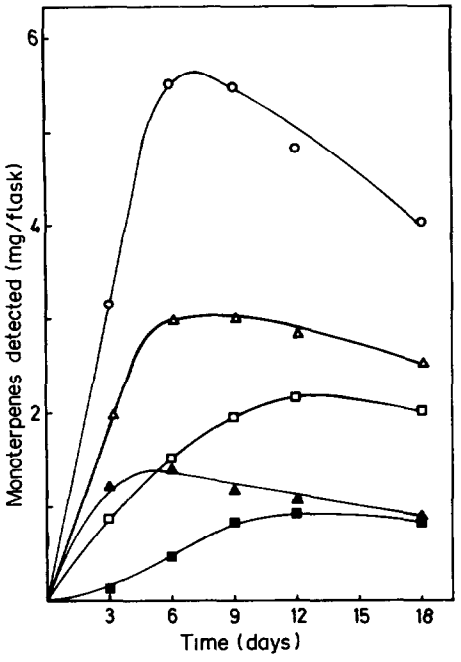

Fig. 2. Accumulation pattern of various monoterpenoids in the medium of cells grown in the absence (▲, ■) and presence (○, △, □) of Miglyol. Aliquots of the Miglyol phase were taken on the days shown. In untreated cells, aliquots of medium were extracted with a small volume of CH_2Cl_2. The flasks contained 8.5 ± 0.3 g (fr. wt) *Thuja* cells at the beginning and 10.5 ± 0.5 g after 18 days. ○——○ terpinolene; △——△/▲——▲, terpinen-4-ol; □——□/■——■, methoxy-*p*-cymen-8-ol.

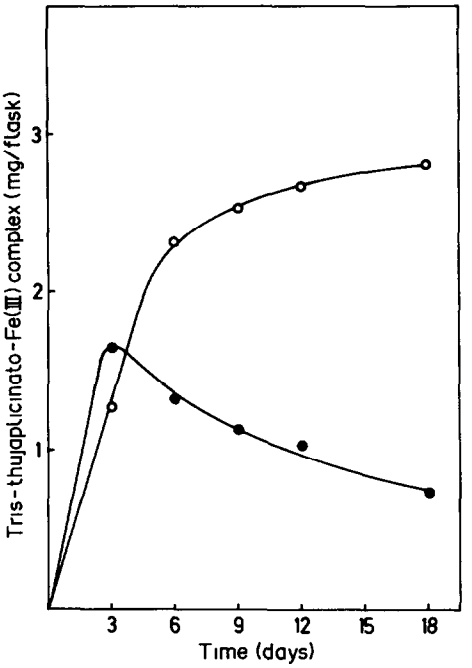

Fig. 3. Accumulation pattern of Tris-thujaplicinato-Fe(III)-complex in the medium of cells grown in the absence (●——●) and presence (○——○) of Miglyol. Conditions as in Fig. 2.

Experiments to use the *Thuja* cells in continuous culture systems are underway.

EXPERIMENTAL

Cell material Establishment and maintenance of the *T. occidentalis* cell cultures have been described recently [1]. For growth and productivity measurement 8.5 g cells (fr. wt) were inoculated into 70 ml of B5-medium [4]. During a period of 18 days the fr. wt increased by 1.5–2.5 g. In the case of altered media the first analysis was performed after three transfers into the new medium All experiments were repeated twice in triplicate.

Analytical procedures An aliquot of the culture medium was extracted with CH_2Cl_2 at the appropriate time. In the case of Miglyol treated cells 10 ml Miglyol were added to the culture medium. When necessary the medium was quickly separated from the cells and an aliquot of the Miglyol phase was taken. Extraction of the H_2O phase with CH_2Cl_2 showed that more than 95% of the monoterpenes accumulated in Miglyol. The Miglyol- and the CH_2Cl_2-extract were analysed by GLC as described [1]. Calibration curves for α-pinene, terpinolene and terpinen-4-ol were used for quantification. The Tris-thujaplicinato-Fe(III)-complex was measured at 540 nm (ε = 4200). The curve was calibrated with chemically synthesized material [1]. Sucrose and glucose were determined enzymatically with a Boehringer test set. Pi was measured by the molybdate method [6]

Chemical identification. GLC/MS was performed with 15 m × 0.23 mm i.d. fused silica capillary columns coated with the methylsilicon phase DB-1. Conditions: Temp. prog. 70°–300°, 6°/min at 300° isothermal; carrier gas He, 0.7 bar This was coupled to an AEI/MS-30 spectrometer via an open split connection Spectra were recorded at 24 eV in combination with the data system AEI DS50. ^1H NMR spectra were recorded at ambient temperature on a Bruker WM 400 spectrometer operating at 400. 14 MHz and locked to the ^2H resonance of the $CDCl_3$ solvent.

REFERENCES

1. Witte, L., Berlin, J., Wray, V., Schubert, W., Kohl, W., Höfle, G. and Hammer, J. (1983) *Planta Med.* **49**, 216.
2. Beiderbeck, R. (1982) *Z. Pflanzenphysiol.* **108**, 27.
3. Bisson, W., Beiderbeck, R. and Reichling, J. (1983) *Planta Med.* **47**, 164.
4. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) *Exp. Cell Res.* **50**, 148.
5. Knobloch, K.-H., Beutnagel, G. and Berlin, J. (1981) *Planta* **153**, 582.
6. Ames, B. R. and Dubin, D. T (1960) *J. Biol. Chem.* **23**, 769.