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Feedback regulation of β-thujaplicin production and formation of its methyl ether in a suspension culture of *Cupressus lusitanica*☆

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Abstract

Suspension cell cultures of *Cupressus lusitanica* produce β-thujaplicin, a tropolone found mostly in Cupressaceae heartwood. The factors controlling β-thujaplicin accumulation in this cell culture system were investigated. Initial cell density of the cultures did not affect β-thujaplicin levels, though initial addition of β-thujaplicin suppressed its de novo production. When β-thujaplicin accumulation reached a certain level (ca. 40 mg/l) in the medium, the cultures seemed to cease β-thujaplicin production. However, β-thujaplicin productivity was restored when the β-thujaplicin-containing medium was exchanged for fresh medium; the formation of 2-methoxy-6-(methylethyl)cyclohepta-2,4,6-trien-1-one, an isomer of methylated β-thujaplicin, in medium was also observed. These results suggest that β-thujaplicin synthesis was regulated by product feedback mechanism in this cell line, and that excess accumulation of β-thujaplicin is relieved by conversion of β-thujaplicin to its methyl ether. © 2002 Published by Elsevier Science Ltd.

Keywords: β-Thujaplicin; Monoterpene; Methylation; Plant cell culture; Cupressaceae; Product inhibition; *Cupressus lusitanica*

1. Introduction

Plant cell cultures provide an attractive way to obtain highly valuable plant-derived products, such as flavors, fragrances, alkaloids, pigments, and pharmaceuticals, which are expensive to synthesize chemically and occur naturally only at low concentrations. Recently, the production of valuable plant secondary metabolites have been achieved, but in most cases the yields have been too low for commercial production (Discosmo and Misawa, 1996).

β-Thujaplicin **1**, known as hinokitiol in Japan (Nozoe and Katsura, 1944), is responsible for the durability and resistance of trees against insect attacks and fungal decay, both of which are characteristic of the heartwood of most species in the Cupressaceae family (Dev, 1989; Haluk and Roussel, 2000). Its broad antimicrobial spectrum (Yamaguchi et al., 1999) led to its wide use in cosmetics, clinical products, and in other applications. Most of this compound is derived from sawdust of *Thujopsis dolabrata* in Japan. However, β-thujaplicin **1** content is very low (0.02% on sawdust basis), and a complicated purification system is required because many components are intermingled with the essential oil (Okabe et al., 1990). Therefore, plant cell culture is one reasonable approach to achieving commercial production of β-thujaplicin.

From such a viewpoint, the production of β-thujaplicin **1** using cell cultures was investigated. The optimum culture conditions for cell growth and β-thujaplicin **1** production of our callus line induced from *Cupressus lusitanica* were previously reported (Inada et al., 1993; Itose and Sakai, 1997). Biological activities were also demonstrated in an ethyl acetate extract from elicitor-treated *C. lusitanica* suspension cell culture, suggesting that the extract becomes a valuable source of bioactivity (Yamaguchi et al., 1999). Though several different cell cultures from different Cupressaceae species have been derived for β-thujaplicin **1** production (Witte et al., 1983; Fujii et al., 1995; Ono et al., 1998), establishment of a mass production system has not yet been achieved. It is generally difficult to create a method for industrial-scale manipulation of tree culture cells because of their

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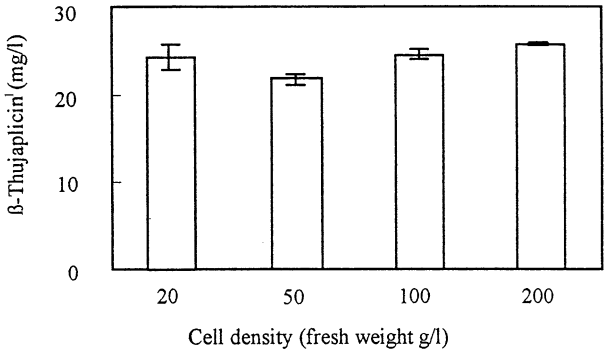


Fig. 1. Effect of initial cell density on β-thujaplicin **1** production in a suspension culture of *C. lusitanica* cells.

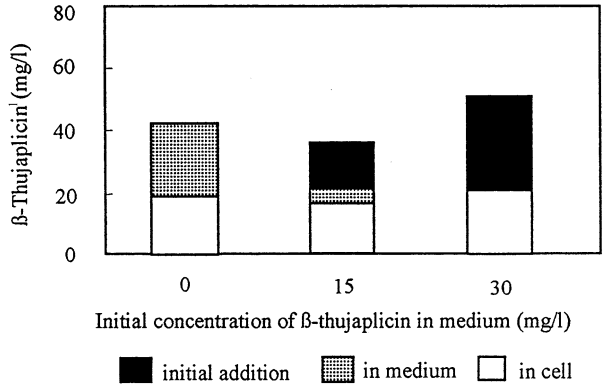


Fig. 2. Effect of initial β-thujaplicin **1** concentration on β-thujaplicin **1** production in a suspension culture of *C. lusitanica* cells.

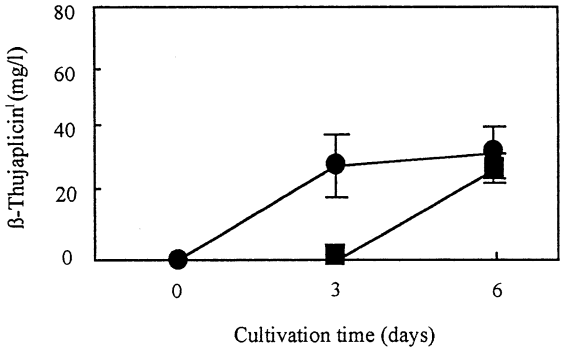


Fig. 3. Effect of medium exchange on β-thujaplicin **1** production in medium in a suspension culture of *C. lusitanica* cells. Medium exchanged on day 3 (■), vo exchange (●).

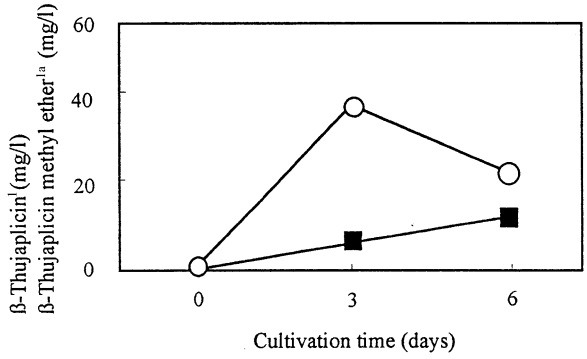
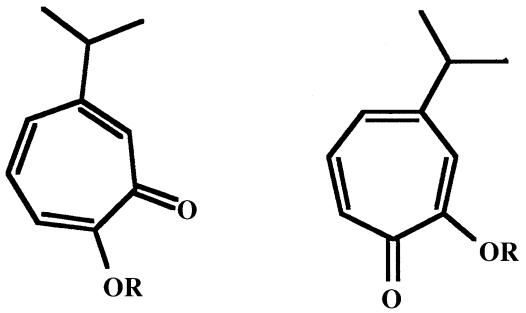


Fig. 4. Time course change of β-thujaplicin **1** (○) and its methyl ether **1a** (■) concentrations in medium of a suspension culture of *C. lusitanica* cells.

slow growth and sensitivity to culture stress (Sanada et al., 2000). During optimization of culture conditions nutrients, elicitor addition, etc.), it was found that one of the regulating factors of β-thujaplicin production in *C. lusitanica* cell culture was product inhibition by β-thujaplicin **1** itself.

2. Results and discussion

Cell density is an important parameter for successful cell culture. In *C. lusitanica* cell suspension cultures, all cell densities tested showed the same level of β-thujaplicin in the medium after 5-day incubations, as shown in Fig. 1. This result suggested that the cells perceived the concentration of β-thujaplicin **1** in the medium and that the cells controlled its accumulation level depending on its concentration. In order to prove this hypothesis, the effect of β-thujaplicin **1** addition to the culture on the final level of β-thujaplicin **1** accumulation was determined. Fig. 2 shows that the total concentrations of artificial and de novo β-thujaplicin **1** in the medium were at the same level regardless of the initial doses of β-thujaplicin **1**. Consequently, secretion of β-thujaplicin **1** into the medium was reduced, whereas the level of β-thujaplicin **1** accumulation in cells remained the same upon its exogenous addition. That is, the initial addition of β-thujaplicin **1** to the culture suppressed its de novo production, and this cell line regulated both intra- and extra-cellular concentrations of β-thujaplicin. Based on the above findings, it was expected that removal of β-thujaplicin **1** from the *C. lusitanica* culture system would stimulate further production and excretion of β-thujaplicin **1** into the medium. To reduce β-thujaplicin **1** concentration, medium exchange was performed and the accumulation level was compared to that in the system without medium exchange. As shown in Fig. 3, the accumulation of β-thujaplicin **1** in the medium was limited to ca. 30 mg/l without medium exchange. However,



1, R=H
1a, R=CH_3

1b, R=CH_3

after the medium was changed to fresh medium on day 3, the cells further produced and excreted ca. 30 mg/l of β-thujaplicin **1** from day 3 to day 6. Therefore, β-thujaplicin productivity can be improved by reducing β-thujaplicin **1** concentration in the medium. This result also suggested that β-thujaplicin production depended on the concentration of β-thujaplicin **1** in the medium, as mentioned above.

In many cases, β-thujaplicin **1** concentration in this cell line reached maximum levels on days 3–6, then decreased over the next several days (Zhao et al., 2001). On the other hand, an unknown peak, which continually increased for all incubation periods, was observed upon HPLC analysis of the medium. A compound corresponding to the peak was preliminary purified and identified as β-thujaplicin methyl ether **1a** by comparison to a chemically synthesized sample. Though Furuya and coworkers reported the biotransformation of β-thujaplicin into glycosylation products by cultured cells of *Eucalyptus perriniana* (Furuya et al., 1997), the natural occurrence of methylated β-thujaplicin **1a**/**1b** has not yet been reported. Another possible methyl derivative from β-thujaplicin, **1b**, was not observed even at trace levels on GC–MS analysis, suggesting the existence of a methylating enzyme, which has a high substrate specificity. Neither **1a** nor **1b** was found inside of the *C. lusitanica* cells. The concentration of **1a** increased while β-thujaplicin concentration was reduced after day 3 (Fig. 4). Moreover, an intentional addition of at least up to 20 mg/l of **1a** to the culture did not affect the accumulation of β-thujaplicin **1** (Fig. 5), whereas addition of β-thujaplicin **1** itself suppressed β-thujaplicin **1** production as described above. Though toxicity of β-thujaplicin **1** against plant cells is not well known, there are some reports showing its toxicity against microorganisms and animal cells (Nakagawa and Tayama, 1998). The excess β-thujaplicin **1** was converted to its methyl derivative **1a** probably to reduce its toxicity against *C. lusitanica* cells.

In conclusion, the accumulation of β-thujaplicin **1** in *C. lusitanica* cell cultures was regulated by a product feedback mechanism, and excess β-thujaplicin **1** was excluded from the feedback system by methylation of β-thujaplicin to give **1a**. Therefore, removal of β-thujaplicin **1** from the medium will lead to a more efficient culture system for β-thujaplicin **1** production.

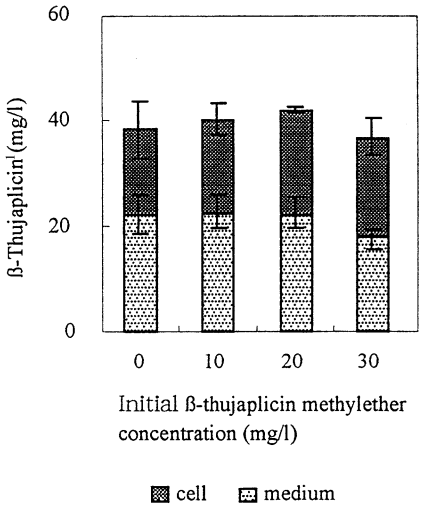


Fig. 5. Effect of concentration of intentionally added **1a** on β-thujaplicin **1** production in a suspension culture of *C. lusitanica* cell.

3. Experimental

3.1. Callus and suspension culture for cell maintenance

Callus cultures of *C. lusitanica* were maintained on Gamborg B5 medium (Gamborg et al., 1968) supplemented with 20 g/l sucrose, 0.01 μM BAP, 10 μM NAA, and 2.7 g/l Gel-rite at pH 5.5 for more than 10 years, as described previously (Itose and Sakai, 1997). The callus cells of *C. lusitanica* were transferred to suspension cultures and maintained in IS-1 medium (Itose and Sakai, 1997), which is a modified Gamborg B5 medium containing 0.01 mM Fe(II) in the original B5 liquid medium, at 25 °C in the dark in a rotary shaker (70 rpm).

3.2. β-Thujaplicin production and determination

C. lusitanica cells grown in a 500 ml flask with 120 ml IS-1 medium were separated from the medium by filtering samples through Miracloth with suction, and a portion of the residue was transferred to a 50 ml flask with 10 ml IS-2 medium (Itose and Sakai, 1997) containing 0.25 mM Fe(II) and major inorganic nutrients at 0.1 strength of the original B5 medium. To stimulate β-thujaplicin production, 1 ml of an elicitor, soln. (18 g/l), was added to this culture (Sakai et al., 1994). The cells were incubated at 25 °C in the dark in a rotary shaker (70 rpm) for 6 days. After the incubation, cells separated by Miracloth were homogenized in a mortar with a pestle. Homogenized cells and medium were separately extracted twice with EtOAc. The amount of β-thujaplicin **1** in the extract was determined by reversed-phase HPLC (Endo et al., 1988). The amount of β-thujaplicin **1** that accumulated was shown as a quantity per 1 l of the culture system.

3.3. Identification of *1a*

Authentic β-thujaplicin methyl ether isomers **1a** and **1b** were synthesized by the methylation of β-thujaplicin **1** obtained from Wako Chemical Co. (Osaka, Japan) with diazomethane. Each isomer (**1a** and **1b**) was purified by Si-gel CC using Et_2O–hexane (1:1), and subsequently identified by NMR spectroscopic analysis.

A crude Et_2O extract was prepared from the above 10 l culture medium on day 3 after elicitation. The Et_2O layer was extracted with 1N HCl aq. The acidic aq. phase was neutralized and extracted with Et_2O. After preliminary separation by reversed-phase prep. LC, β-thujaplicin methyl ether **1a** was identified using GC–MS by comparison with the synthesized sample.

*3.3.1. 2-Methoxy-6-(methylethyl)cyclohepta-2,4,6-trien-1-one (**1a**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.23 (6H, d, $J=6.8$ Hz, H-9, 10), 2.78 (1H, *m*, $J=6.8$ Hz, H-8), 3.92 (3H, *s*, H-8), 6.65 (1H, *d*, $J=10.0$ Hz, H-3), 6.79 (1H, *dd*, $J_{5,7}=1.7$ Hz, $J_{4,5}=11.1$ Hz, H-5), 7.01 (1H, *dd*, $J_{3,4}=10.0$ Hz, $J_{4,5}=11.1$ Hz, H-4), 7.18 (1H, *d*, $J=1.7$ Hz, H-7); ^{13}C NMR (100 MHz, $CHCl_3$-d_1): δ 22 (C-9, 10), 38 (C-8), 56 (C-11). 111 (C-3), 129 (C-5), 131 (C-4), 134 (C-7), 157 (C-6), 164 (C-1), 179 (C-2); positive ion EI–MS: *m*/*z* (%) 51 (56), 77 (56), 79 (54), 91 (82), 105 (100), 135 (92), 147 (64), 149 (52), 178 (M^+, 84).

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