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Improved Culture Conditions for the Production of β-Thujaplicin by Suspension Cell Cultures of *Cupressus lusitanica*

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Abstract

An improvement in the production of β-thujaplicin (hinokitiol) by the *Cupressus lusitanica* suspension cell cultures is described. For cell growth, a sufficient supply of nutrients was necessary but Fe(II) had an inhibitory effect. The cells grew well in a modified Gamborg B5 medium (IS-1) containing Fe of 0.01mM which is one tenth of that in the original B5 medium. On the contrary, insufficient inorganic nutrients and excess Fe were effective for the β-thujaplicin production. Thus, the cells accumulated 22mg of β-thujaplicin/g dry cell weight in 3 days after elicitation, when 20g/*l* glucose was used as a carbon source in the other modified Gamborg B5 medium (IS-2) which contained 0.25mM Fe and major nutrients at 0.1 strength of the original B5 medium. The glucose dose can be greatly reduced without seriously affecting the β-thujaplicin production level, if a proper osmotic pressure regulator is present in the medium.

1. Introduction

β-Thujaplicin (hinokitiol), one of the wood tropolones with a seven-membered ring and an isopropyl side chain, is specifically contained in the heartwood of trees which belong to the family Cupressaceae [1]. Due to its broad antimicrobial spectrum, the compound has been utilized as an ingredient of toiletry products such as hair tonic, tooth paste, skin cream and so on. Recent attention has been denoted to the antibacterial effects of β-thujaplicin against methicillin-resistant *Staphylococcus aureus*, an important cause of hospital-acquired infection [2]. Madar *et al.* recently observed that glucosylated thujaplicin derivatives were formed in the bark of *Cupressus sempervirens* (Italian cypress) when the tree was infected by a fungal pathogen [3]. Such observation seems reasonable because we have found that formation of β-thujaplicin is elicited in *C. lusitanica* callus cultures by fungal elicitors [4, 5].

The production of β-thujaplicin by means of suspension cell culture has been performed by Witte *et al.* [6, 7], Hirose *et al.* [8], and Fujii *et al.* [9] using cells from different Cupressaceae species, but its productivities reported so far were rather low. For example Fujii *et al.* [9] reported that the highest hinokitiol content in their *Thujopsis dolabrata* cells was 422 μg/g fresh cell weight that is roughly equivalent to 4-5 mg/g dry cell weight. We therefore attempted to improve the productivity of β-thujaplicin in a suspension culture based on the previously established callus culture of *C. lusitanica* [4]. We also aimed to decrease sugar concentration in the culture medium so that we may use this culture system for tracer experiments [10] using the rather expensive labeled glucose and for a large production of β-thujaplicin.

2. Materials and Methods

2.1 Plant hormones

6-Benzylaminopurine (BAP) and kinetin were used as cytokinins in a concentration range between 0.01 and 0.5μM. As auxins, naphthylacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) were used in concentrations 1 to 50μM.

2.2 Cell cultures and culture condition

Callus cultures of *C. lusitanica* Miller have been maintained on Gamborg B5 medium [11] supplemented with 20 g/*l* sucrose, 0.01μM BAP, 10μM NAA at pH5.5 for more than 7 years as described previously [4, 5]. The callus cells of *C. lusitanica* were transferred to suspension cultures and maintained at 25°C in the dark on a rotary shaker (70 rpm) in modified B5 liquid media containing plant hormones and 20 g/*l* sucrose.

2.3 Cell growth determination

At the end of growth experiments, the cells were collected on a glass fiber filter, washed and dried in a vacuum oven at 70°C for 18-20 h. Cell growth index was defined as W/W_o: here W and W_o denote cell dry

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Table 1.
Effect of nutrient concentrations on the growth of suspension culture cells of *C. lusitanica*.

Medium	Concentrations (% of Gamborg B5 midium)				Growth index (W/W_o)*
	Major nutrients	Minor salts	Vitamins	Fe(II)	
B5	100	100	100	100	1.21
IS-1a	10	10	10	10	1.58
IS-1b	10	10	10	50	1.52
IS-1c	10	10	50	10	1.81
IS-1d	10	50	10	10	1.77
IS-1e	50	10	10	10	1.84
IS-1	100	100	100	10	2.07

* W and Wo denote cell dry weights before and after the cultivation for 10 days, respectively. Each value represents average of at least duplicates. Standard deviations were less than 0.15 except for the B5 medium where color of most cultures changed to yellowish brown in several days of incubations.

weights before and after the cultivation experiments, respectively.

For obtaining time course of cell growth, cell suspension cultures were initiated by transferring *ca.* 0.4 g of callus cells to 10m*l* of the IS-1 medium (**Table 1**) in a 50 m*l* flask and the cells were harvested on days 4, 10, 14, 18, and 22 for determining the growth index.

2.4 Production, extraction and determination of β-thujaplicin

To produce β-thujaplicin, 0.5 m*l* of an elicitor solution was added at the beginning of subculture to the production medium (IS-2). The elicitor was prepared from yeast extract (Difco lab.) by the fractional precipitation with aqueous ethanol of different concentrations [5]. A fraction soluble in 60% ethanol and precipitated with 80% ethanol was employed in the present experiments.

After proper periods of incubation time cultured cells were homogenized in a mortar with a pestle and extracted twice with ethyl acetate. The extract was analyzed by HPLC on μBondasphere 5μ C_4-100A column (Waters) with 25 % (v/v) MeOH containing 0.2 % $CuSO_4$ as eluent with a flow rate of 0.5m*l*/min. The detection was carried out at 325 nm. Identification of β-thujaplicin produced was described previously [4, 5].

2.5 Effect of sugar on the production of β-thujaplicin

Elicitor was added to culture cells of *C. lusitanica* incubated in IS-2 medium (10m*l*) containing glucose, sucrose, galactose, mannose, fructose, sorbitol, mannitol or their mixtures as carbon source. After 3 days of incubation, cells were homogenized and extracted with ethyl acetate, and the extract was analyzed by HPLC as described above.

3. Results and Discussion

3.1 Growth studies in suspension cultures

To establish suspension cultures of *C. lusitanica* cells, we preliminary used the Gamborg B5 medium [11] on which *C. lusitanica* callus cells exhibited fast growth [4]. In the B5 liquid medium, however, the suspension cells grew very slowly and cell browning was often observed during incubation of several days. We, at first, investigated the effect of plant hormone compositions on cell growth. A combination of 0.01 μM BAP and 10 μM NAA was the optimum for the cell growth in the range of experimental conditions. However, the suspension cells still tended to exhibit the browning at sometimes with this combination of plant hormones. Use of kinetin and 2,4-D provided no beneficial effect on the growth of *C. lusitanica* suspension cultures.

Therefore, the concentrations of major nutrients, minor mineral salts, Fe(II) and vitamins in B5 medium were changed for obtaining better growth of *C. lusitanica* suspension cells, using the above-mentioned combination of plant hormones. As shown in **Table 1**, cell growth after 10 days of incubation was slightly improved by decreasing nutrient concentrations in the IS-1a medium as compared with growth in the original B5 medium. But the growth was still insufficient due to nutrient deficiency. Moderate growth in IS-1c, IS-1d, and IS-1e media containing Fe at 0.1 strength (0.01mM) and one of the other nutrient groups at half strength of the B5 medium suggest that certain amounts of hormones, major nutrients and vitamins are necessary but excess Fe is detrimental to cell growth of the *C. lusitanica* suspension culture. Thus, a modified B5 medium (IS-1) containing full strength nutrients but 0.1 strength (0.01mM) Fe

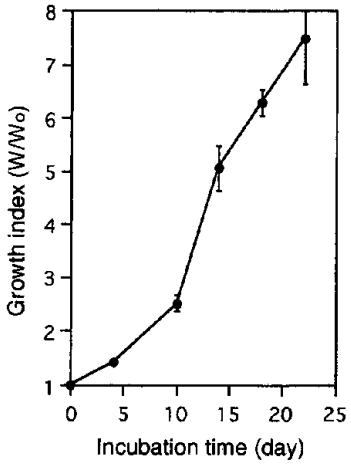

Fig. 1 Growth of suspension culture cells of *C. lusitanica* in the IS-1 medium.
W_0 and W denote cell dry weights at the start of experiment and sampling times, respectively. Each value represents the average of three replicates and vertical lines represent the standard deviation of replicates.

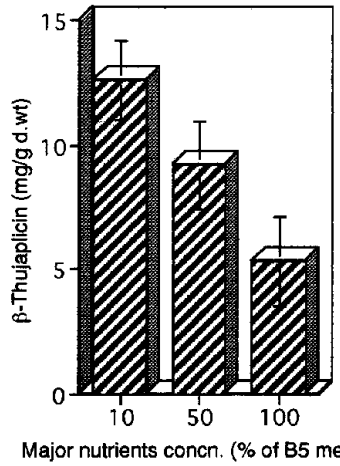

Fig. 2 Effect of major nutrient concentrations on the β-thujaplicin production by suspension cell cultures of *C. lusitanica* in 4 days after the elicitor addition. Medium compositions were the same as the B5 medium except major nutrient concentration. An elicitor (9 mg) was added at the start of experiments. Each value represents the average of at least three replicates and vertical lines represent the standard deviation of replicates.

supplemented with 0.01 μM BAP, 10 μM NAA and 20 g/*l* sucrose was adopted as a growth medium.

The time course of the suspension cell growth of *C. lusitanica* in IS-1 medium exhibited a typical growth curve with an induction period for about seven days and a subsequent logarithmic phase between days 10 and 18 as shown in **Fig. 1.** Cell dry weight increased to about 4.5-fold and 7.5-fold in 14 and 22 days, respectively. Almost no β-thujaplicin was produced by *C. lusitanica* cultures in this medium.

3.2 Production of β-thujaplicin in suspension cultures

C. lusitanica produced β-thujaplicin when an elicitor was added to its suspension cultures at the beginning of subculture, as observed for callus cultures of the same species [4, 5]. In order to determine the optimum elicitor concentrations for maximal β-thujaplicin accumulation, different amounts between 4.5 and 27 mg of the elicitor were added to 10m*l* aliquots of cell suspensions. Cultures were harvested on day 3 after the addition of the elicitor and analyzed for β-thujaplicin content. Maximal accumulation of β-thujaplicin was observed with the 9 mg of the elicitor addition (data not shown).

To obtain maximum production of β-thujaplicin, concentrations of major nutrients and Fe in B5 medium were varied. It was observed that a higher concentration of major nutrients resulted in a lower production of β-thujaplicin (**Fig. 2**) and that maximum β-thujaplicin production was obtained at 0.25mM Fe which is 2.5 times higher than in the ordinary B5 medium (**Fig. 3**). These results suggest that a sufficient supply of major nutrients retard the onset

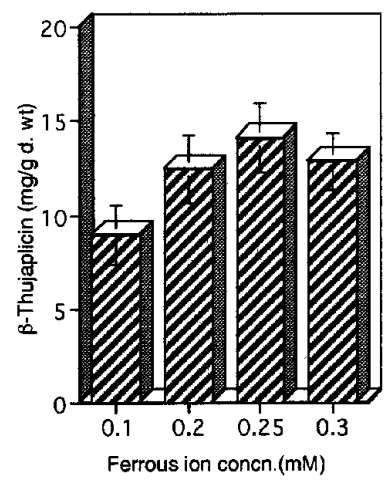

Fig. 3 Effect of Fe(II) concentrations on the production of β-thujaplicin by *C. lusitanica* suspension cells in 3 days after the elicitor addition.
Medium compositions were the same as the B5 medium except Fe(II) concentration. An elicitor (9 mg) was added at the start of experiments. Each value represents the average of duplicates and vertical lines represent the error of replicates.

of secondary metabolite productions and that β-thujaplicin formation was promoted by the increase of Fe, though excess Fe has a detrimental effect on cell growth. Thus, a modified B5 medium (IS-2) containing major nutrients at 0.1 strength of the original and 0.25mM Fe supplemented with 0.01 μM BAP, 10 μM NAA and 20g/*l* sucrose was adopted as a production medium.

Time course of the production of β-thujaplicin by *C. lusitanica* cells cultured in IS-2 medium is shown in

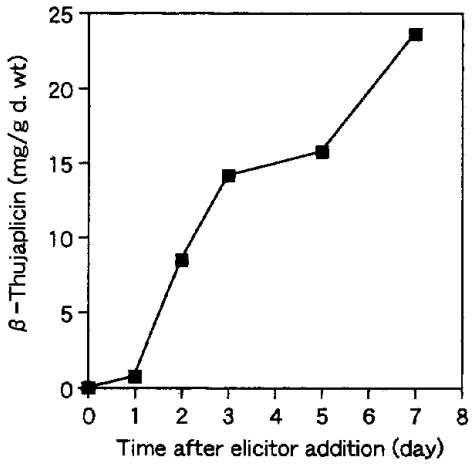

Fig. 4 Time course of the β-thujaplicin accumulation by *C. lusitanica* suspension cell cultures in the IS-2 medium.
An elicitor (9 mg) was added at the start of experiments. Each value represents the average of three replicates.

Fig. 4. The suspension cells produced β-thujaplicin of about 15 and 24 mg /g dry cell weight within 3 days and 7 days, respectively. The contents seem to be much higher than that produced by *T. dolabrata* cells after 7 days of cultivation (0.422 mg/g fresh cells) [9]. This elicitation phenomenon was so reproducible that biochemical study can be performed with the *C. lusitanica* cells to get information about the pathway of β-thujaplicin biosynthesis in short culture time [12].

3.3 *Effects of sugar type and sugar concentration on the production of β-thujaplicin*

As mentioned above, β-thujaplicin can be produced by *C. lusitanica* suspension cells in 2-3 days after elicitor addition in the IS-2 medium. This fact suggests that such a large amount of carbon source such as 20 g/*l* sucrose is not necessary for β-thujaplicin production because the cells did not grow to a discernible extent in this period of incubation. It was therefore expected that sugar concentration in the production medium can be decreased to a great extent while keeping the β-thujaplicin productivity relatively high. For this purpose we studied the effect of concentrations of various carbohydrates on the β-thujaplicin productivity in *C. lusitanica* suspension cells. The results are shown in **Table 2**. Glucose was the most favorable carbon source among the different mono- and disaccharides employed for the β-thujaplicin production. Thus, *ca.* 22 mg of β-thujaplicin/g dry cell weight was produced when 20 g/*l* glucose was used in the production medium. A decrease or increase of glucose from this concentration resulted in the reduced production of β-thujaplicin. It is generally recognized that raising the initial sugar levels

Table 2.
Effect of sugar on the production of β-thujaplicin by *C. lusitanica* suspension culture cells.

Sugar	Concn. (g/*l*)	β-Thujaplicin* (mg/g dry wt. ±s. d.)
Glucose	30	7.4±1.7
	20	21.6±0.6
	15	10.5±0.9
	10	7.4±1.3
	5	4.0±0.3
Sucrose	40	10.2±2.3
	20	15.7±2.1
Galactose	20	15.6±0.8
Mannose	20	11.2±1.1
Fructose	20	11.4±0.4

* Determined after 3 days of incubations. Each value represents average of at least duplicates.

Table 3.
Effect of sugar and sugar alcohol mixtures on the production of β-thujaplicin by *C. lusitanica* suspension culture cells.

Concn. (g/*l*)			β-Thujaplicin* (mg/g dry wt. ±s. d.)
Glucose	Sorbitol	Mannitol	
5	15	0	21.6±1.0
1	19	0	9.5±0.7
0	20	0	6.9±0.8
0	15	0	5.3±0.4
5	0	15	18.0±3.1
1	0	19	14.8±0.9
0	0	20	6.7±0.4
0	0	15	5.7±0.2

* Determined after 3 days of incubations. Each value represents average of at least duplicates.

leads to an increase in secondary metabolite production [13]. Kim *et al.* [14] also reported the effect of sugar concentration on the production of taxol and related taxanes in *Taxus brevifolia* cell cultures. In this connection, the use of 40 g/*l* sucrose which roughly corresponds to 20 g/*l* glucose in molar concentration resulted in the low level of the β-thujaplicin production probably because sucrose was hydrolyzed quickly to glucose and fructose in the *C. lusitanica* culture.

Sugar is likely to play two important roles in the suspension cultures as a carbon source and as an osmotic pressure regulator, though the latter role in the synthesis of secondary metabolites is not clearly understood. Thus we investigated the effect of sugar alcohols on the level of β-thujaplicin. As shown in **Table 3** sugar alcohols (sorbitol and mannitol) alone were not suitable for the production of β-thujaplicin because they are not effective carbon sources even though they act as osmotic pressure regulators.

However, the level of β-thujaplicin production increased significantly in the media containing mixtures of sugar alcohols and small amounts of glucose. The β-thujaplicin productions in mixtures of 5g/*l* glucose and 15g/*l* sorbitol (or mannitol) were almost the same as that in 20g/*l* glucose, indicating that 75 % of the glucose in the medium can be replaced by sugar alcohols. The level of β-thujaplicin obtained in a mixture of 1g/*l* glucose and 19g/*l* mannitol was 14. 84 mg/g cell dry weight, which is a level comparable to that produced in 20 g/*l* of sucrose.

It is worth noting that glucose concentration in the IS-2 medium can be reduced to one-fourth without affecting the level of the β-thujaplicin production, if a proper osmotic pressure regulator is present in the medium. Even if the glucose dose was decreased to one-twentieth, the β-thujaplicin production was still 68 % as compared with the culture using 20 g/*l* glucose (see **Table 2** and **3**). Decrease in sugar concentration makes it easier to perform tracer experiments using expensive labeled glucose. Results of tracer experiments to clarify the pathway of β-thujaplicin biosynthesis using ^{14}C- and ^{13}C-labeled glucose will be published elsewhere.

Acknowledgments

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