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ORIGINAL ARTICLE

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Cell growth and nutrient uptake by cell suspensions of *Cupressus lusitanica*

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Abstract Conditions for cell growth of suspension cultures of *Cupressus lusitanica*, which has high β-thujaplicin productivity, were studied. The medium that provided the highest growth rate was IS-1 medium (pH 5.5), modified from Gamborg B5 medium containing 32 mM of total nitrogen. Its NO_3-N/NH_4-N ratio was 30:2. The maximum growth represented a 25-fold increase over the initial biomass on a fresh weight basis after 30 days of culture in this medium. The highest cell growth was obtained with an initial pH of 3.5–5.5, but the pH of the medium settled to about pH 4.0 from any of the initial pH values in this report. The cells cultured under this condition were able to produce a high level of β-thujaplicin.

Key words β-Thujaplicin · Plant cell culture · *Cupressus lusitanica* · Nutrient uptake · Cell growth

Introduction

β-Thujaplicin, known as hinokitiol in Japan, is responsible for providing trees with durability and resistance against insect attacks and fungal decays, which are characteristic of the heartwood of most species in the Cupressaceae family.[1–4] Most β-thujaplicin is obtained from the sawdust of *Thujopsis dolabrata* in Japan, but the content in sawdust is low (0.02%).[5] On the other hand, plant cell cultures provide an attractive method for obtaining valuable plant-derived products (e.g., flavors, fragrances, colorants, pharmaceuticals) that are expensive to synthesize chemically and that occur naturally only at low concentrations.[6] Recently, there has been some significant success in the production of important plant secondary metabolites; but in most cases the yields of secondary metabolites are too low for commercial production.[6–8] Therefore, plant cell culture is a reasonable means of commercial production of β-thujaplicin, for which the demand is expected to increase to prevent deforestation.

We have investigated the production of β-thujaplicin with cell cultures of *Cupressus lusitanica* (Mexican cypress). We have already reported that our callus line exhibited strong ability to produce β-thujaplicin, and that an elicitor addition stimulated the accumulation of β-thujaplicin in the callus,[9,10] though a trace quantity of β-thujaplicin was observed under the growth condition. Some of the biological activities of β-thujaplicin were demonstrated in ethyl acetate extracts from an elicitor-treated *C. lusitanica* suspension cell culture,[11] suggesting that the ethyl acetate extract of *C. lusitanica* cells is a valuable bioactive source. In a previous paper, it was shown that a sufficient supply of nutrients and reduced Fe(II) concentration were necessary for cell growth in a suspension culture. However, we also reported that reduced inorganic nutrients and excess Fe(II) were effective for β-thujaplicin production.[12] Though several cell cultures from Cupresaceae families have been studied for β-thujaplicin production,[13–15] the establishment of a mass production system is still ongoing because it is difficult to perform industrial manipulation on plant cells induced from trees because of their slow growth speed and their sensitivity to culture stress.[15]

Therefore, a suspension cell culture system, which enables mass production in a mill, should be established using the *C. lusitanica* cell line. In this article, the effects of a variety of nutrients and pH levels on the cell growth in the culture were investigated to determine the optimum conditions for suspension cultures of *C. lusitanica* cells.

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Materials and methods

Cell cultures and culture conditions

Callus cultures of *C. lusitanica* were maintained on Gamborg B5 medium[16] supplemented with sucrose 20 g/l, benzylaminopurine 0.01 μM, 1-naphthaleneacetic acid 10 μM, and Gel-rite 2.7 g/l at pH 5.5 for more than 10 years as described previously.[12] The callus cells of *C. lusitanica* were transferred to suspension cultures and maintained in IS-1 medium, which is a modified Gamborg B5 medium containing 0.01 mM Fe(II), one-tenth of that in the original B5 liquid medium, at 25°C in the dark on a rotary shaker (70 rpm).[12] The initial pH of the medium was adjusted with dilute HCl or NaOH solution.

Cell growth determination

The fresh cell weight was determined after filtering the samples from the medium through Miracloth (Calbiochem-Novabiochem, San Diego, CA, USA) with suction. The cell growth rate was defined as W/W_0, where W_0 represents the cell weight before cultivation and W is the cell weight after cultivation.

Nutrient composition determination

The initial concentrations of nitrate-nitrogen (NO_3-N) and ammonium-nitrogen (NH_4-N) in the culture medium were controlled by the doses of KNO_3 and $(NH_4)_2SO_4$, respectively. The ammonium, nitrate, and residual sugar concentrations in the medium were determined using an F-kit ammonia, F-kit nitrate, and F-kit sucrose/glucose/fructose (Boehringer Mannheim, Indianapolis, IN, USA), respectively. The inorganic phosphate concentration in the medium was determined by the ascorbic acid method.[17]

β-Thujaplicin production and determination

Cupressus lusitanica cells grown in a 100-ml flask with 30 ml of IS-1 medium[12] were separated from the medium by filtering the samples through Miracloth with suction. Cells (4.5 g) were then transferred to a 100-ml flask with 30 ml IS-2 medium[12] containing 0.25 mM Fe(II) and major inorganic nutrients at 0.1 strength of the original B5 medium. To stimulate β-thujaplicin production, 3 ml of an elicitor solution (18 g/l)[9] was added to this culture. The cells were incubated at 25°C in the dark on a rotary shaker (70 rpm) for 6 days. After incubation the cells separated by Miracloth were homogenized using a mortar and pestle. The homogenated cells and medium were extracted twice with ethyl acetate. The β-thujaplicin content in the extract was determined by Endo's method.[18]

Results and discusssion

Effect of initial medium composition and concentration on cell growth

To obtain the optimum medium composition for cell growth, the effects of the initial concentrations of nitrogen sources and phosphate on cell growth were investigated. The cell growth was determined at day 14 because obvious growth was observed after the induction period in the preliminary experiments.

Effect of the nitrate nitrogen/ammonia nitrogen ratio and total nitrogen concentration on cell growth

The total nitrogen concentration and the NO_3-N/NH_4-N ratio clearly affect the growth of the cultured plant cell.[1,19–22] To determine the optimum conditions, the cell growth rates of *C. lusitanica* suspension cultures were measured on day 14 of the culture at various total concentrations of nitrogen sources and the NO_3-N/NH_4-N ratios. As shown in Fig. 1, the cell growth of *C. lusitanica* was affected by the NO_3-N/NH_4-N ratio, and the maximum fresh weight was obtained at a ratio of 30:2, which is the original ratio in the Gamborg B5 medium. Our cell line may have adapted to the B5 medium because it has been maintained in B5 medium for more than 10 years. The cells were brown and grew slowly when incubated at a ratio of 8:24, with the NH_4-N concentration being higher than that of NO_3-N.

The effect of the total nitrogen concentration was investigated in concentrations ranging from 16 to 320 mM. The

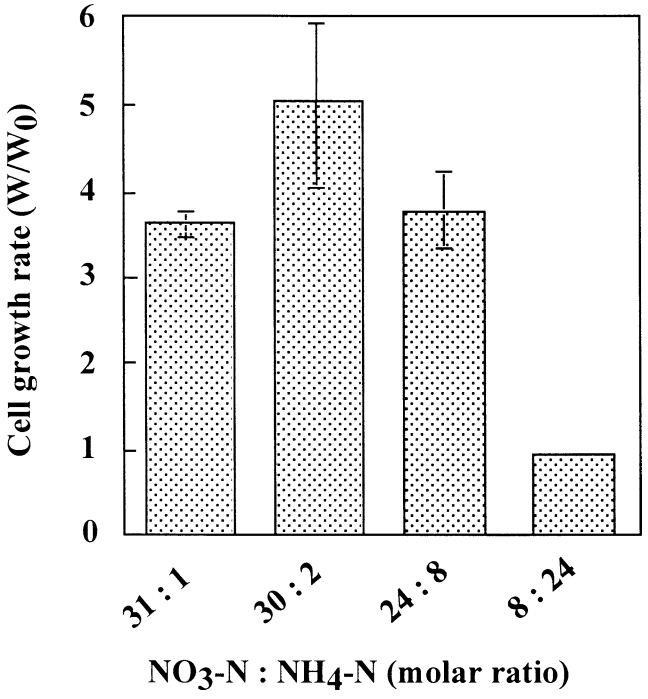

Fig. 1. Effect of the NO_3-N/NH_4-N ratio on cell growth in a suspension culture of *Cupressus lusitanica* cells after a 14-day cultivation. Error bars show standard deviations

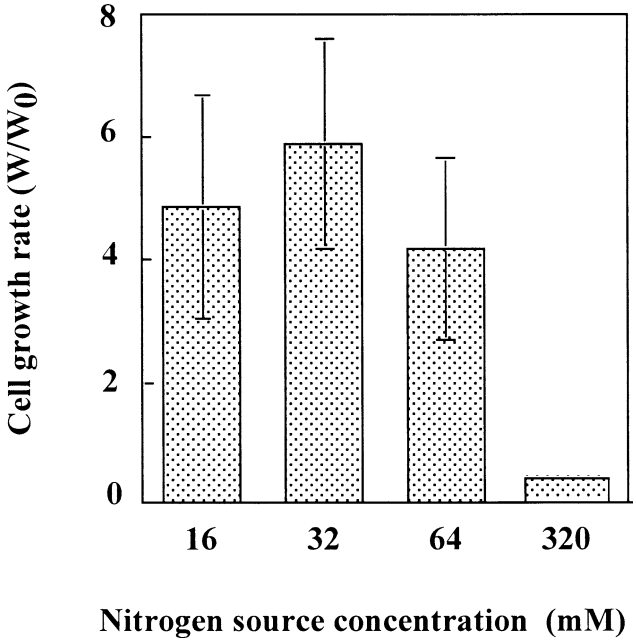

Fig. 2. Effect of nitrogen concentration on cell growth in a suspension culture of *C. lusitanica* cells after a 14-day cultivation. Error bars show standard deviations

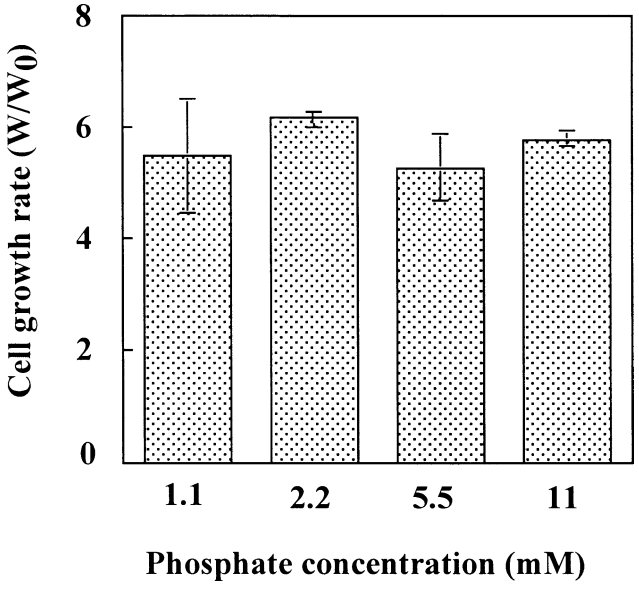

Fig. 3. Effect of phosphate concentration on cell growth in a suspension culture of *C. lusitanica* cells after a 14-day cultivation. Error bars show standard deviations

maximum fresh weight was observed at a 32 mM total nitrogen concentration, with the NO_3-N/NH_4-N ratio maintained at 30:2 (Fig. 2). This nitrogen concentration is the same as the B5 original medium nitrogen concentration that was used for cell line maintenance. Increasing the nitrogen source to 320 mM had the effect of turning the cell color brown and stopping all cell growth. It is possible that the cell cannot endure a high nitrogen concentration, which causes high osmotic pressure.

Effect of phosphate concentration on cell growth

The phosphate concentration also determines the growth of the cultured plant cells.[23,24] The effect on cell growth of an initial phosphate concentration higher than that of the original B5 medium (1.1 mM) was also investigated. The cell growth at 14 days was not affected by any of the initial phosphate concentrations examined in this study (Fig. 3).

These optimum concentrations of nitrogen and phosphate sources for cell growth were equal to those of the Gamborg B5 medium used for preliminary experiments. Because this cell line had been maintained for about 10 years in the original B5 agar medium, it was suggested that adaptation or selection of the cells that were well suited to the culture medium occurred. Based on to this idea, screening the cell line for the best growth may also be considered.

Nutrient uptake and cell growth

Cell growth profile

A 25-fold increase in fresh weight was observed after 30 days of culture in the medium selected by the above experiments (Fig. 4). On a dry weight basis, a 13-fold increase was observed after 25 days of culture in this medium (Fig. 4). The cell growth profile showed a lag phase from 0 to 5 days and growth was maintained throughout the experimental period until day 30. No cell growth was observed on a dry weight basis for days 25–30, though the fresh cell weight increased.

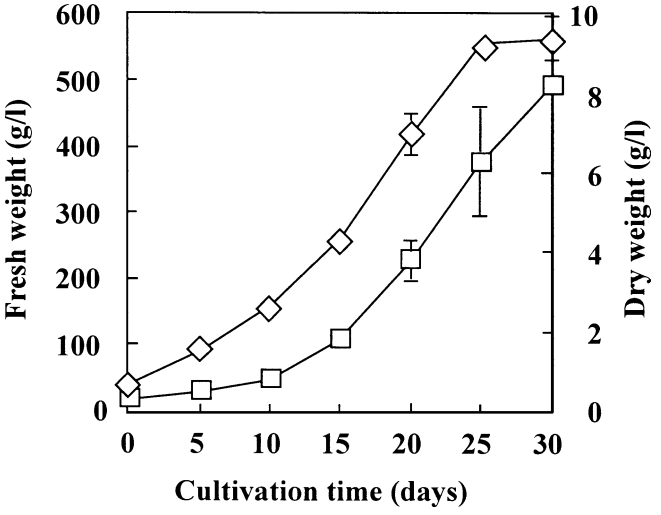

Fig. 4. Growth of *C. lusitanica* cells in a suspension culture in IS-1 medium. Error bars show standard deviations. *Squares*, fresh weight, *diamonds*, dry weight

Consumption of nitrogen sources

The ammonium ions in the medium were consumed rapidly during the first 5 days and were depleted by day 10 (Fig. 5).

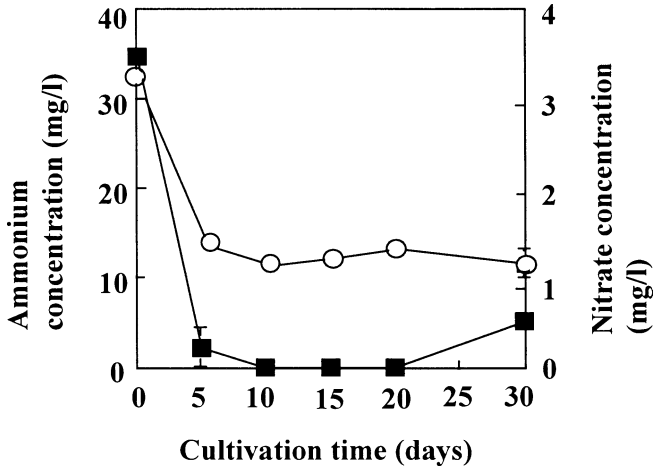

Fig. 5. Changes in nitrate and ammonium concentrations in the culture medium during suspension cultivation of *C. lusitanica* cells. Error bars show standard deviations. *Circles*, Nitrate; *squares*, ammonium

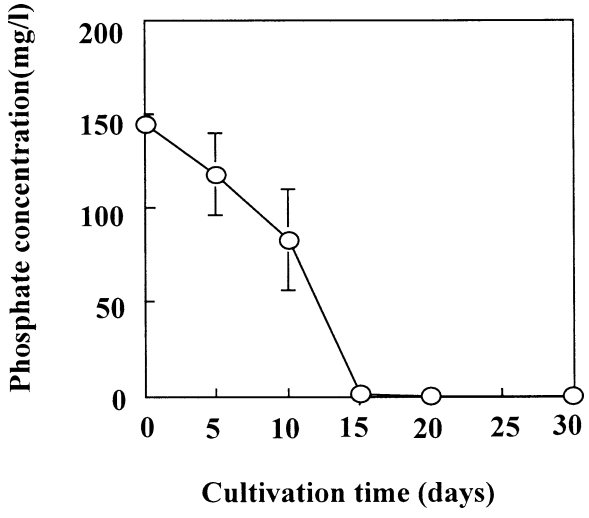

Fig. 6. Changes in phosphate concentration in the culture medium during suspension cultivation of *C. lusitanica* cells. Error bars show standard deviations

The nitrate concentration of the medium had also decreased rapidly by day 5, but nitrate 1.3 mg/l remained at the end of the 30-day cultivation. Therefore, cell growth might increase if ammonium was added on days 5–10 to maintain the NO_3-N/NH_4-N ratio at 30:2, which was the best condition for cell growth (Fig. 1). On days 25–30 the concentration of ammonium ions in the medium slightly increased, suggesting possible autolysis of old cells.

Consumption of phosphate

The cells kept growing during the entire experimental period until day 30 even though the phosphate in the medium was depleted by day 15 (Fig. 6). Because phosphate is essential for DNA synthesis, cell division might stop by day 15, and cell elongation alone might occur after the phosphate depletion. Pepin et al. also proposed that the depletion of residual ammonium and phosphate might limit cell division.[22] Therefore, the addition of phosphate after day 15 may facilitate cell division.

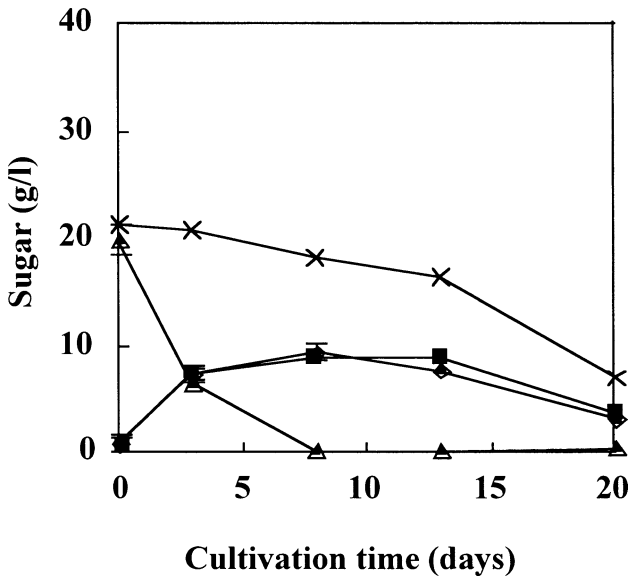

Fig. 7. Changes in residual sugar concentration in the growth medium for a suspension culture of *C. lusitanica* cells. Error bars show standard deviations. *Diamonds*, glucose; *squares*, fructose; *triangles*, sucrose; *crosses*, total weight of glucose, fructose, and sucrose

Consumption of carbohydrates

Sucrose was hydrolyzed rapidly during the first 8 days. In contrast, glucose and fructose appeared in the medium (Fig. 7). Almost no uptake of sugars was found during days 0–5, and there was little change in total sugar concentration on days 5–10. Thereafter, a rapid decrease in sugar was observed between days 15 and 25, which was the logarithmic growth phase of this cell culture system. Sugar was completely taken up into the cells by day 25. Cell growth may be increased by adding sugar during the latter term of cultivation, which would improve cell wall synthesis.

Though the growth profile in Fig. 4 showed an induction period in which little growth on a weight basis and little consumption of the sugar were observed (Figs. 5, 6), phosphate and nitrogen as raw materials of nucleic acid and protein were intensely absorbed during this period. Because a considerable portion of the dry weight would be cell walls in plants, especially in woody plants, it was suggested that the cell population increased by active cell division during the early phase of the culture and construction of the cell wall was mainly carried out during the latter half of the incubation period when sugar consumption was intensified (Fig. 7). These time-course changes in the major nutrients left in the medium suggested that not only the volume and the weight of the cell but also the cell population and cytoplasm quantity may be necessary for growth evaluation during further research of cultured tree cells. The histological observations are of interest for verifying this idea. On the

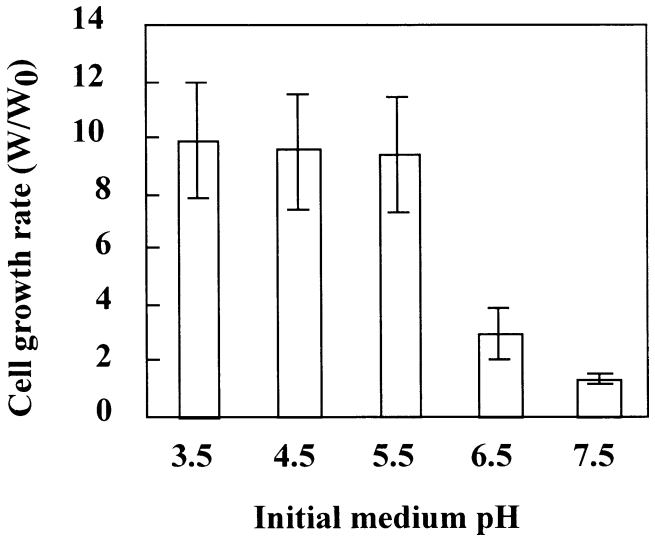

Fig. 8. Effect of the initial pH of the growth medium for a suspension culture of *C. lusitanica* cells. Error bars show standard deviations. Growth rate was measured at day 14 on a fresh weight basis

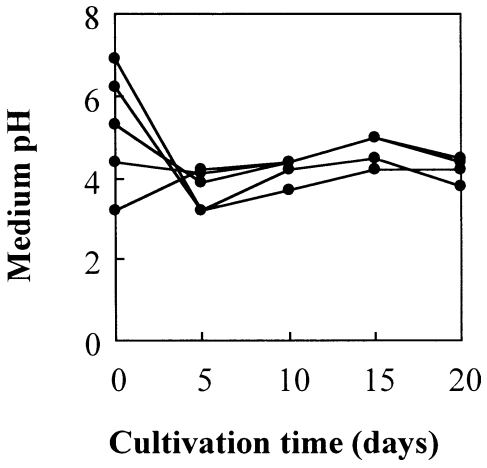

Fig. 9. Changes in the pH of the medium from various initial precultivation pH values of *C. lusitanica* cells in a suspension culture

other hand, to ficilitate automated and efficient β-thujaplicin production, a continuous supply of nitrogen and phosphate is necessary to maintain conditions whereby cell wall thickening is avoided because such thickening would not contribute to the production of the β-thujaplicin.

Medium pH

Effect of initial medium pH on cell growth

The effect of the initial pH of the medium on the growth of *C. lusitanica* suspension cells was investigated. The highest cell growth at day 14 was obtained with an initial pH of 3.5–5.5 (Fig. 8). At an initial pH in 6.5–7.5, cell growth was lower and cell color was changed to light brown, suggesting that the pH in this range may stimulate secondary metabolite production.[15]

Time course of medium pH

The time course changes of various initial medium pH levels were investigated. The pH levels of the medium at day 0 were adjusted to 3.5, 4.5, 5.5, 6.5, and 7.5 but were changed to 3.7, 4.8, 5.4, 6.4, and 7.2, respectively, after autoclaving (121°C, 20 min) for sterilization. Regardless of the different initial pH values, all samples showed a pH of about 4 on day 3, which was the first sampling time (Fig. 9). This indicated that *C. lusitanica* cells have a buffering capacity and can change their extracellular pH from a range of 3.5–7.5 to the suitable pH region around 4.0. Such a trend was also observed during anthocyanin production with strawberry cell suspension cultures.[25] One of the reasons the pH 3.5 showed a higher growth index might be that this pH was close to the optimum pH at which *C. lusitanica cells* adjusted by themselves.

Table 1. β-Thujaplicin production of *C. lusitanica* cells grown in IS-1 medium

Cells	β-Thujaplicin production (mg/fr.w.g)
From IS-1 medium[a]	0.35
From solid medium[b]	0.37

Induction time for β-thujaplicin production was 6 days after transferring cells to IS-2 medium
[a] Cells were cultured in IS-1 medium for 4 weeks
[b] Cells were cultured on B-5 solid medium[9] for 4 weeks

β-Thujaplicin production

In our previous report,[12] cells were grown on solid medium as callus and transferred to the liquid medium to start β-thujaplicin production. The β-thujaplicin production level of the cells grown as a suspension culture in the liquid modified medium examined above was measured to clarify the effect of this growth condition on the following production stage. *C. lusitanica* cells grown in the liquid medium for 4 weeks were transferred to the liquid β-thujaplicine production medium reported previously.[12] The highest β-thujaplicin production was 40 mg/l (0.35 mg/fresh weight g) at day 6 after the induction of β-thujaplicin production, which is equivalent to the production level of the cells prepared from the callus state,[12] as shown in Table 1. These results suggest that this cell line grown under these conditions has the same ability to produce β-thujaplicin as does the callus culture.

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