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ORIGINAL ARTICLE

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Effect of major inorganic nutrients on β-thujaplicin production in a suspension culture of *Cupressus lusitanica* cells

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Abstract The optimum conditions for β-thujaplicin production in a *Cupressus lusitanica* cell suspension culture were investigated. The conditions required for β-thujaplicin production were clearly different from the conditions for cell growth. The initial phosphate concentration and pH did not affect β-thujaplicin production. A total nitrogen source concentration higher than 3.2 mM suppressed production due to the presence of the ammonium ion. β-Thujaplicin production was observed at 95 mg/l without adding the ammonium ion to the medium. Strict control of major inorganic nutrients was not necessary to produce β-thujaplicin. This finding seems to be favorable for future automated production of β-thujaplicin in commercial cell culture plants.

Key words β-Thujaplicin · Plant cell culture · *Cupressus lusitanica* · Ammonium ion

Introduction

Plant cell culture is a promising area of research that could provide high value plant-derived products such as flavors, fragrances, colorants, and pharmaceuticals, particularly those that are expensive to synthesize chemically and those that naturally occur only at low concentrations.[1] Some significant improvements in the production of important plant secondary metabolites have already been reported,[1–3] but in most cases it is difficult to obtain enough yield of cell biomass and secondary metabolites for viable commercial production. Therefore, the optimization of cell growth and secondary metabolite production in the plant cell culture system is required for each species and each objective of plant cell culture.

We have reported that our cell line of cultured *Cupressus lusitanica* (Mexican cypress) has good ability to product β-thujaplicin,[4,5] a strong phytoalexine and therefore responsible for the natural durability of some Cupresaceae wood species.[6] Although several cell cultures from the Cupresaceae family have been studied for the purpose of β-thujaplicin production,[7–9] establishing a mass production system is still ongoing, as it is difficult for plant cells induced from trees to be manipulated industrially because of their slow growth and their sensitivity to culture stress.[9] As a first step in targeting the commercial base production of β-thujaplicin, the optimum composition of major inorganic nutrient for cell growth of a suspension culture of *C. lusitanica* was investigated previously.[10] Some reports have suggested that the medium's composition suitable for cell growth was not the best one for maximizing the production of secondary metabolites and vice versa.[11–13] We therefore explored various media for use as a suspension culture to determine which one was optimal for cell growth and for β-thujaplicin production. In particular, our study focused on the difference in the optimum compositions of major inorganic nutrients for β-thujaplicin production.

Materials and methods

Cell line maintenance

Callus cultures of *C. lusitanica* have been maintained on Gamborg B5 medium[14] supplemented with sucrose 20 g/l, 6-benzylamino purine 0.01 μM, 1-naphthalene acetic acid 10 μM, and Gel-rite 2.7 g/l at pH 5.5 for more than 10 years, as described previously.[15] The initial pH of the medium was adjusted with HCl or NaOH.

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β-Thujaplicin production and determination

The callus cells of *C. lusitanica* were transferred to suspension cultures and maintained in a 100-ml flask with 30 ml 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. It was kept at 25°C in the dark on a rotary shaker (70 rpm)[15] prior to the β-thujaplicin production experiments. *C. lusitanica* cells grown in a 100-ml flask were separated from the medium by filtering the samples through Miracloth (Calbiochem-Novabiochem, San Diego, CA, USA) with suction; and 1.5 g of the cells were transferred to a 50-ml flask with 10 ml IS-2 medium containing 0.25 mM Fe(II) and major inorganic nutrients at one-tenth the strength of the original B5 medium.[15] To stimulate β-thujaplicin production, 3 ml of an elicitor solution (18 g/l)[5] was added to this culture. The initial concentrations of nitrate ion, ammonium ion, and phosphate ion were controlled by the doses of KNO_3, $(NH_4)_2SO_4$, and Na_2HPO_4, respectively. The initial pH of the medium was adjusted with HCl or NaOH. The cells were incubated at 25°C in the dark on a rotary shaker (70 rpm) for 5 days. After incubation, the cells were separated by Miracloth filtration and were homogenized in a mortar with a 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.[16] The amount of β-thujaplicin that had accumulated was reported as the quantity per liter of culture system.

Results and discussion

The optimum conditions for the growth of *C. lusitanica* cells in a suspension culture were investigated previously.[10] Unfortunately, the medium conditions optimized for cell growth were sometimes different from those that produced maximum production of secondary metabolites. The total nitrogen source concentration, nitrate/ammonium ion ratio, phosphate concentration, and pH were especially important factors for controlling the secondary metabolism.[11–13,17,18] In a previous investigation of suspension cultures of our cell line,[15] the growth medium was somewhat modified, and the authors found that increased Fe and decreased major inorganic nutrient concentrations improved β-thujaplicin accumulation. However, the active component in major inorganic salts was not specified, and a pH investigation was not performed. Therefore, the effects of the total nitrogen, nitrate/ammonium ion ratio, phosphate concentration, and pH on β-thujaplicin production were examined in the current study.

Effect of total nitrogen source concentrations on β-thujaplicin production

Incubation of *C. lusitanica* cell at various concentrations of the nitrogen (N) sources was initiated with addition of the elicitor, and the accumulation of β-thujaplicin was determined on day 5. The initial nitrate/ammonium ion ratio was kept at 30:2. As shown in Fig. 1, β-thujaplicin production was suppressed at high N concentrations (>3.2 mM), and production of 45 mg/l was observed even without any major N sources. According to Srinivasan and Ryu,[11] shikonin production did not improve well in an N-rich medium, though the cell biomass was increased. The effect of the nitrate/ammonium ion ratio on the production of β-thujaplicin was also investigated. The accumulation of β-thujaplicin was obviously decreased by a high nitrate/ammonium ion ratio, and the best accumulation was observed in the medium containing no ammonium ion (Fig. 2). On the other hand, the nitrate concentration did not affect β-thujaplicin production in the medium containing no ammonium ion (Fig. 3). Based on these results, it was concluded that the effect of the total N source observed above was almost completely due to the ammonium ion concentration. According to Kinooka et al., the presence of ammonium ions in the medium resulted in enhanced superoxide dismutase activity in the hairy root cell cultivation of horseradish.[19] Because β-thujaplicin production in our cell line was initiated through the typical signal transduction system including oxidative burst,[20] one of the possibilities of this suppression might be inhibition of signal transduction.

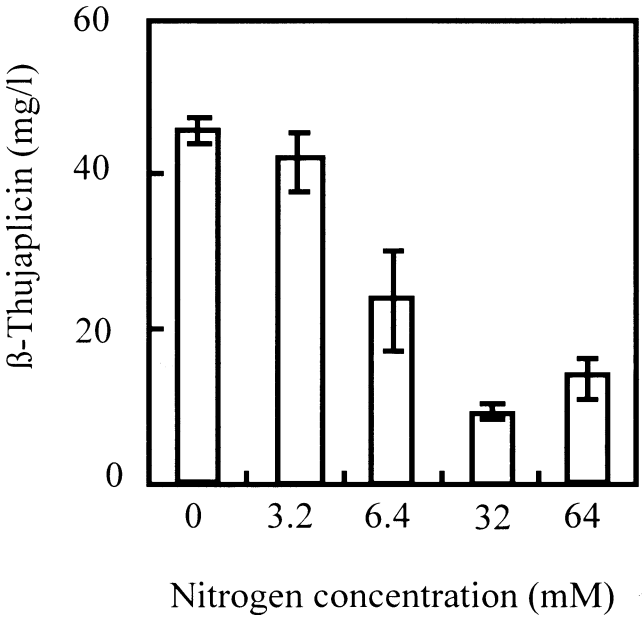

Fig. 1. Effect of total nitrogen concentration on β-thujaplicin production in a suspension culture of *Cupressus lusitanica* cells. The nitrate/ammonium ion ratio was 30:2. Error bars show standard deviations

Effect of phosphate concentration on β-thujaplicin production

Phosphoric acid is a nutrient indispensable for plant growth. For secondary metabolite production, it was reported that ginsenoside saponin production by cell cultures of *Panax notoginseng* was affected by the initial phosphate concentration in the medium.[21] However, the level of β-thujaplicin

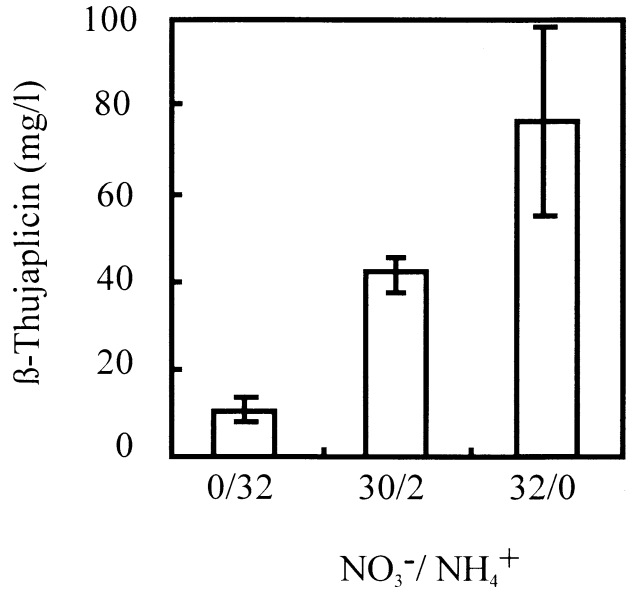

Fig. 2. Effect of the nitrate ammonium ion ratio on β-thujaplicin production in a suspension culture of *C. lusitanica* cells. Total nitrogen concentration was 3.2 mM. Error bars show standard deviations

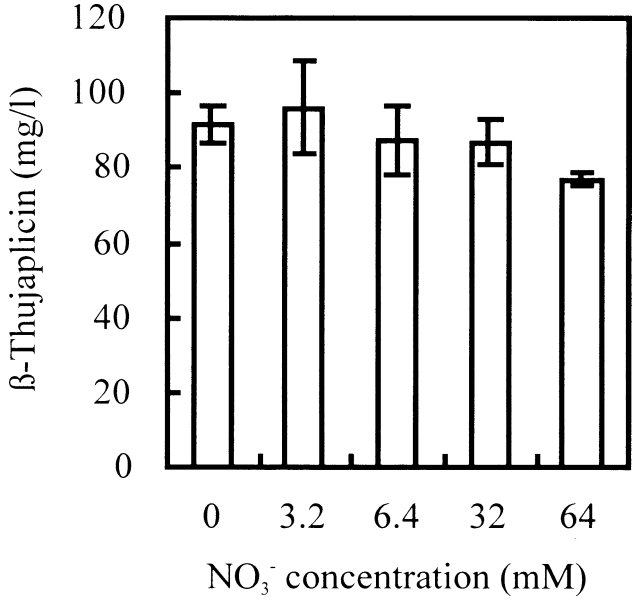

Fig. 3. Effect of the nitrate ion concentration on β-thujaplicin production in a suspension culture of *C. lusitanica* cells. No ammonium ion source was added. Error bars show standard deviations

accumulation of this *C. lusitanica* cell culture system did not change at phosphate concentrations in the range of 0–2.2 mM, as shown in Fig. 4. Because a significant accumulation was observed even with no phosphate in the medium, it is clear that addition of extracellular phosphate is not necessary for β-thujaplicin production during short incubations (up to 5 days). No cell growth was observed after the elicitation of this *C. lusitanica* cell culture (data not shown), and it was suggested in a previous report that phosphate was required during the cell division phase.[10] Pepin et al.

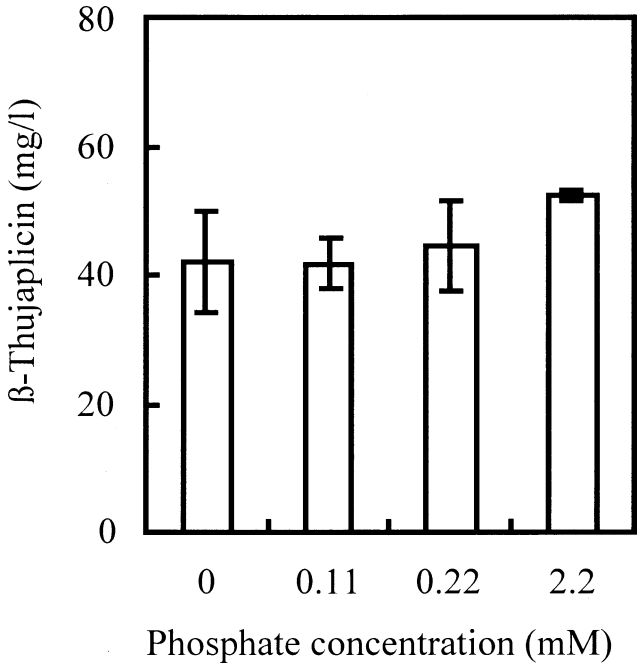

Fig. 4. Effect of initial phosphate concentration on β-thujaplicin production in a suspension culture of *C. lusitanica* cells. Total nitrogen concentration and the nitrate/ammonium ion ratio were 3.2 mM and 30:2, respectively. Error bars show standard deviations

also discussed the possibility that extracellular phosphate availability might limit the cell division in *Vitis vinifera* cell suspension culture.[22] Therefore, extracellular phosphate in the medium has almost not role in β-thujaplicin accumulation during the short period in which cell division does not occur. Itose and Sakai[15] reported improved β-thujaplicin accumulation with our cell line when the major nutrients included in our previous study were used at reduced strength. This improvement was due to the concentration of ammonium ion according to the results shown in Figs. 1 and 2, and there was no effect of phosphate or nitrate.

Effect of medium pH on β-thujaplicin production

The pH of the medium had little effect on the cell growth of *C. lusitanica* cell suspension culture as reported previously, probably because this cell line has a pH-buffering ability that allows it to adjust the medium to a favorable pH.[10] The effect of an initial pH in the range of 3.5–7.5 on β-thujaplicin production was not obvious (Fig. 5). As was observed in the experiment on growth, the initial pH of the medium (3.5–7.5) rapidly settled at about 4.8 until day 3, the first sampling day. In general, the pH of the medium has a great effect on the results of cell culture. It was reported that pH in the alkaline range stimulated production of anthocyanin in a suspension culture of strawberry cells.[23] Our *C. lusitanica* cell suspension culture did not need strict pH control for β-thujaplicin production.

As mentioned above, strict control of the pH and major inorganic nutrients except for ammonium ions were not

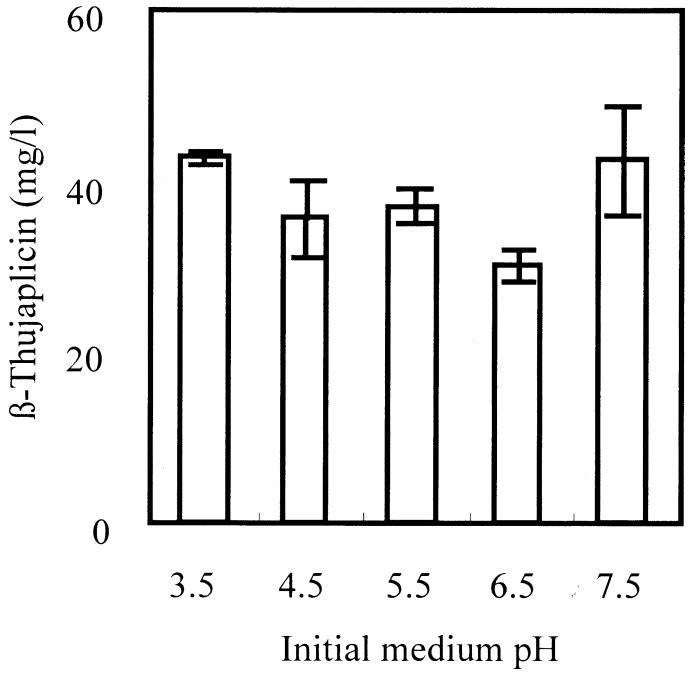

Fig. 5. Effect of initial medium pH on β-thujaplicin production in a suspension culture of *C. lusitanica* cells. The medium was IS-2 medium modified by Itose and Sakai.[15] Error bars show standard deviations

necessary to produce β-thujaplicin in this culture system. However, during the cultivation that was undertaken to obtain cell biomass prior to β-thujaplicin production, the cells completely consumed the ammonium ions in the medium;[10] therefore, no ammonium, which suppressed β-thujaplicin production, remained when the system moved on to the next stage. These properties seem to be favorable for simplifying the future automated production of β-thujaplicin in commercial cell culture plants.

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