Optimization of the Sucrose and Ion Concentrations for Saikosaponin Production in Hairy Root Culture of *Bupleurum falcatum*

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Abstract Saikosaponin productivity was examined in a *Bupleurum falcatum* L. BFHR2 hairy root culture in response to changes in the sucrose content (2~8%), nitrogen content (0~250 mM NH_4NO_3), phosphate content (0~12 mM NaH_2PO_4), and the potassium content (0~87.2 mM KCl) of the culture media. We found that the conditions for maximal saikosaponin production differed from those for optimal root growth. Highest saikosaponin yield was achieved for 8% sucrose, 62 mM NH_4NO_3, 1.2 mM NaH_2PO_4, and 0.5 mM KCl.

Keywords: saikosaponin, hairy root, sucrose, macro-nutrients, productivity

INTRODUCTION

The roots of *Bupleurum falcatum* L., a perennial herb belonging to the *Umbelliferae* family, are widely used in traditional Chinese medicine because of their antiinflammatory, antipyretic, and antitussive properties [1]. Activity of saikosaponin-a, saikosaponin-d, and saikosaponin-c aglycones has been reported in plasma cholesterol reduction [2], analgesia [3], immunoregulation [4], hemolysis [5], cell membrane stabilization [6], allergy attenuation, and virus inactivation [7-10]. More recently, saikosponin has also been associated with the inhibition of melanin synthesizing enzymes [11] and the stimulation of skin collagen synthesis [12]. This leads to the development of saikosaponin-containing cosmetics in Korea and Japan and, consequentially, a growing interest for increased saikosaponin production efficiency from *B. falcatum* roots.

One of the rate-limiting factors in saikosaponin production is the slow growth of the *B. falcatum* roots. Saikosponin content is high in younger (approx. 1 yr) roots, but it decreases as the plant ages [13]. There are considerable variations in growth, morphological characteristics, and saikosaponin content between individual *B. falcatum* L. plants because of genetic variation [14], geographical differences in the climate and the soil [15-19], and varying cultivation methods [20,21].

It has been proposed to develop plant tissue culture methodology capable of satisfying the growing demand for saikosaponin. Adventitious roots and Ri-plasmid-transformed roots have been found to grow sufficiently rapid and to have higher saikosaponin content than native roots [22,23]. Some significant improvements have been made by selecting specific hairy root lines [22] and by exploring variations of the culture conditions, such as alkalinization, addition of exogenous hormones and signal transducers [23-25], and two-step culturing [26] to improve saikosaponin production. It is generally very difficult, however, to obtain a biomass and secondary metabolite yield suited for commercial production. There is hence a strong demand for further optimization of this methodology.

The synthesis of secondary metabolites depends on the physical and chemical culture conditions [27], which, in some cases, differ from those optimal for maximal cell growth. Nitrogen, phosphate, potassium, and sucrose, as well as the nitrate/ammonium ion ratio are important factors for secondary metabolite production [28-31]. Earlier work on *B. falcatum* hairy root cultures has revealed a gross difference in MS [32] media with high salt concentration compared to RCM media, which provides fewer salt, in terms of cell growth and saikosaponin content [25]. The compounds responsible for these improvements were not identified, and the sucrose concentration was not optimized. In the here-presented work, we examined the effects of variations in nitrogen, phosphate, and potassium contents as well as the influence of changes in the sucrose concentration in the media while monitoring cell growth, saikosaponin content, and saikosaponin yield.

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MATERIALS AND METHODS

Subculturing Conditions

All experiments were conducted on the hairy root line BFHR2, which was induced from axenic *B. falcatum* L. plantlets by transformation with *Agrobacterium rhizogenes* strain A_4 as described previously [22]. The hairy roots were shaken (100 rpm) at 25°C with a light/dark cycle of 16/8 h for 20 days. Incubation media was liquid 3RCM, which is modified root culture medium (RCM) [33] containing three times of the regular amount of macroelements in RCM medium. The media contained 3 g/L sucrose but no phytohormones, and the pH was adjusted to 5.8 before autoclaving it at 121°C for 15 min. Root fragments subcultured in fresh media for 5 days were used as inocula for all experiments.

Culture Conditions

The effect of alterations in the culture media on cell growth and saikosaponin content was determined using 3RCM media as the base by adjusting the sucrose concentration to 2, 3, 4, 5, 6, 7, or 8%. The pH of the media was adjusted to 5.8 prior to its sterilization. The cultures were inoculated with 0.5 g fresh weight of hairy roots per 30 mL liquid media in a 100 mL Erlenmeyer flask, which was followed by 6 weeks of dark-culture at 25°C on a rotary shaker (100 rpm). At the sampling time, the fresh weight of the cultured roots was measured after removing the surface water and additional drying for 48 h in a freeze dryer. To determine the effects of the inorganic contents of the 3RCM media on the growth rate and the saikosaponin production, we adjusted nitrogen, phosphate, and potassium using NH_4NO_3, NaH_2PO_4, and KCl, respectively. The tested final concentrations were: NH_4NO_3; 0, 13, 62, or 250 mM, NaH_2PO_4; 0, 0.06, 0.12, 0.36, 1.2, 6, or 12 mM, and KCl; 0, 0.5, 0.87, 2.6, 8.7, 43.6, or 87.2 mM. Inoculations and subsequent incubation were done as described above. At the indicated sampling time, the roots' fresh weight was determined after briefly blotting their surface with filter paper and freeze-drying them for additional 48 h. All experiments were performed simultaneously and in triplicate.

Saikosaponin Analysis

Saikosaponin content was determined by grinding 0.2 mg dried roots followed by three 20 min extractions with 5 mL of 2% NaOH-MeOH (v/v) in an ultrasonic bath. The combined methanol extracts were centrifuged, concentrated by evaporation, and the supernatant filtered through Whatman paper (No. 2, 70 mm diameter). The filtrate was collected and the solute removed by evaporation in a rotary evaporator at 50°C. The remainder was resuspended 1:1 (v/v) in H_2O:ethyl ether and the water phase mixed 1:1 (v/v) with butanol. The organic phase was retrieved, concentrated under N_2 flow, redissolved in 2 mL methanol, and filtered through a 0.45-μm membrane. Ten microliters of the suspension were then injected into a high-pressure liquid chromatography system, consisting of a 602 pump (Waters, USA), a 600 injector, a 486 tunable absorbance detector, and an Autochro-WIN integrator (Younglin, Korea). Analytical separations were carried out using a μ-Bondapak C18 column (Waters, USA, 10 μm, 3.9 × 300 mm) protected with a C18 guard column with a 7:3 (v/v, A) gradient of water:acetonitrile and 3:7 (v/v, B) water:acetonitrile as the mobile phase. Elution was done linearly over 25 min from 100:0 to 0:100 (A:B) followed by a 10 min water wash. The system was re-equilibrated in between runs for 10 min with absolute methanol. The flow rate was maintained at 1.1 mL/min and with detection at 205 nm. Concentrations of saikosaponin-a, -c, and -d (w/w) were determined using external saikosaponin standards (Wako, Japan) with the peak area as the quantitative parameter.

Statistical Analysis

Data sets were compared with ANOVA or Duncan's multiple range tests where $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Optimization of the Media Sucrose Concentration for Saikosaponin Production

We first examined the effects of sucrose concentration (2~8%) in the *B. falcatum* root culture media on the cell growth rate and the saikosaponin content (Fig. 1). Our results show that the fresh weight of root was highest (35.6 g/L) at 4% sucrose with higher concentrations having adverse effects. The dry weight of root, on the other hand, tended to increase throughout the entire range of the sucrose concentrations tested, reaching 6.3 g/L at 8% sucrose. The dry-weight/fresh-weight ratio (× 100) was 7.9 for 2% sucrose, increasing with the sucrose concentration to a maximum of 19.4 at 8% sucrose. We noted some necrosis at concentrations >5%. Lateral root formation was also inhibited (data not shown), and growth was slower than at lower sucrose concentrations.

The finding that hairy roots exposed to media containing higher sucrose concentrations showed reduced growth but increased dry weight may have been related to different cell densities and inconsistent moisture content of cells. The saikosaponin yield was expected to vary accordingly due to osmotic pressure-induced changes in the root cells. A similar result has been reported by Shinivasan and Ryu [34]. Sucrose seems to be a critical factor for changes in the dry weight/fresh weight ratio as it affected the cell volume of *Lithospermum erythrorhizon* cell culture based on its osmotic pressure effects. Kusakari *et al.* [26] have reported that lateral root formation is a crucial step for rapid growth which could be significantly inhibited by elevated sugar concentrations in untransformed root cultures of *B. falcatum.*

Contents of saikosaponin-a, -c, and -d were high at 3.51 ± 0.4%, 1.16 ± 0.5%, and 1.11 ± 0.02%, respec-

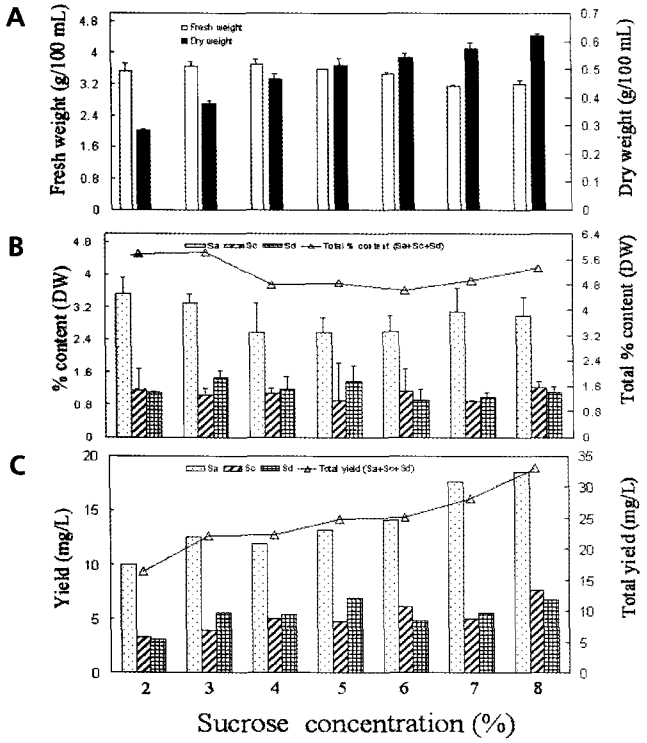

Fig. 1. Effect of alterations in the sucrose concentration (2~8%) on cell growth (A), saikosaponin content (B), and saikosaponin yield (C) of *B. falcatum* BFHR2 hairy roots after 6 weeks of dark culture in 3RCM liquid media at 25°C. Initial inoculation: 0.5 g/flask. Data are means ± standard errors.

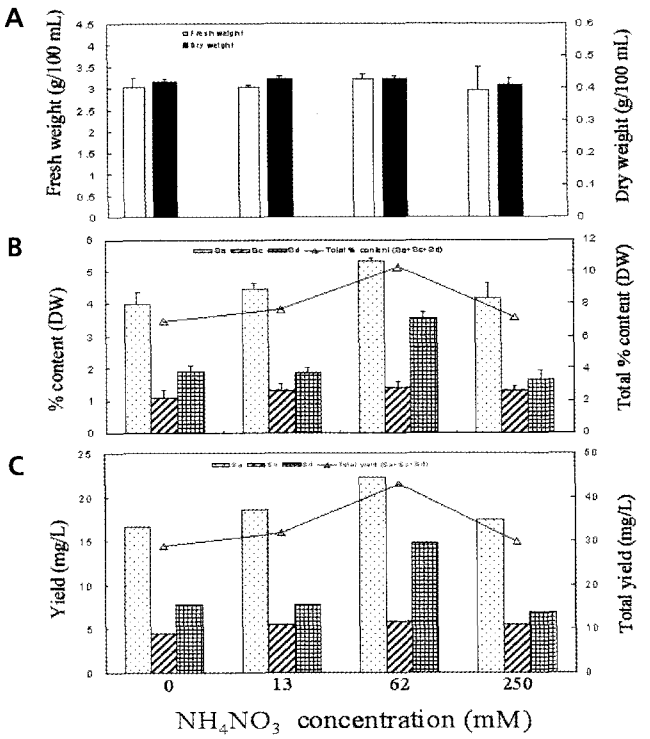

Fig. 2. Effect of total nitrogen content (NH_4NO_3, 0~250 mM) on growth (A), saikosaponin content (B), and saikosaponin yield (C) of *B. falcatum* BFHR2 hairy roots after 6 weeks of dark culture in 3RCM liquid media containing 3% sucrose (25°C). Initial inoculation: 0.5 g/flask. Bars are means ± standard errors.

tively (total: 5.78%) in 2% sucrose, but were minimal at 2.60 ± 0.39%, 1.13 ± 0.55%, and 0.90 ± 0.28%, respectively (total: 4.63%) in 6% sucrose. Maximal production of saikosaponin-a, -c, and -d was 18.59, 7.65, and 6.78 mg/L, respectively at 8% sucrose, with a total of 33.02 mg/L. Hairy root growth and saikosaponin production were balanced by the positive (higher dry weight and saikosaponin yield) and negative aspects (high osmotic stress) of high-sucrose environments. To find the optimum, we therefore opted for a two-step culture procedure, where the sucrose levels can be changed (*e.g.*, an initial low level and a final high level). This approach has previously been used by Kusakari and colleagues on untransformed root cultures of *B. falcatum* [26].

Adjustment of Ion Content in the Culture Media for Optimal Saikosaponin Production

Plants commonly use NH_4^+ and NO_3^- as nitrogen sources, albeit with great variation across species and developmental stage [35]. Absorbed nitrogen is assimilated and utilized in the growth process to produce protein, nucleic acid, amines, chlorophyll, and coenzymes. It is well established that NH_4^+/NO_3^- added to culture media has a positive effect on the production of substances essential to growth. It is hence possible to derive optimal conditions regarding the type and concentration of the nitrogen source to achieve maximal growth and strong production of commercially valuable substances [36]. In this study, we supplement the 3RCM media with NH_4NO_3 at 0, 13, 62, and 250 mM, respectively. Our data show that hairy root growth peaked at 13 and 62 mM with 0.43 ± 0.01 g (fresh weight: 3.03 ± 0.05 g and 3.21 ± 0.12 g, respectively). Growth rapidly declined at NH_4NO_3 concentrations exceeding 625 mM (data not shown). Saikosaponin-a, -c, and -d contents reached 5.4 ± 0.06% (22.33 mg/L), 1.35 ± 0.39% (5.87 mg/L), and 3.29 ± 0.21% (14.85 mg/L, total 10.04% or 43.05 mg/L) at 62 mM (Fig. 2). From these results, we concluded that the optimal ammonium level for saikosaponin production was 62 mM.

Phosphoric acid is indispensable to cell growth in wild plants as well as in *in vitro* cell. It has been demonstrated in cell cultures of *Panax notoginseng* that production of gensenoside saponin, a secondary metabolite, varied according to the phosphate concentration in culture media [37]. Experiments with *Cupressus lusitanica* cell cultures, on the other hand, have shown no relation of the β-thujaplicin level to the supplied phosphate concentration [28]. Work in our lab has yielded data proving MS media, which has a higher phosphate content (KH_2PO_4, 1.25 mM) than RCM media (NaH_2PO_4, 0.1 mM), to be optimal for growth of *B. falcatum* hairy roots cultures. The same media performs, however, considerably poorer than RCM media in terms of saikosaponin production [25].

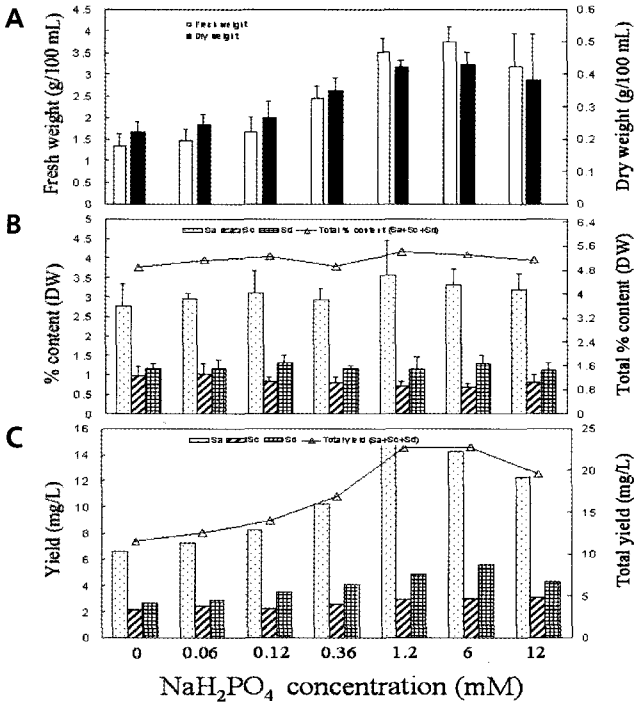

Fig. 3. Effect of phosphate content (NaH_2PO_4, 0~12 mM) on growth (A), saikosaponin content (B), and saikosaponin yield (C) of *B. falcatum* BFHR2 hairy roots after 6 weeks of dark culture in 3RCM liquid media containing 3% sucrose (25°C). Initial inoculation: 0.5 g/flask. Bars are means ± standard errors.

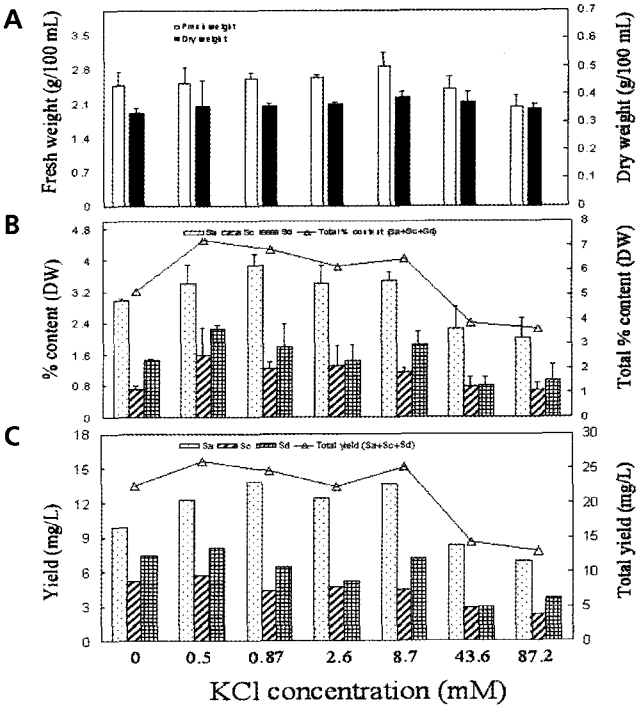

Fig. 4. Effect of potassium content (KCl, 0~87.2 mM) on growth (A), saikosaponin content (B), and saikosaponin yield (C) of *B. falcatum* BFHR2 hairy roots after 6 weeks of dark culture in 3RCM liquid media containing 3% sucrose (25°C). Initial inoculation: 0.5 g/flask. Bars are means ± standard errors.

As shown in Fig. 3, varying the concentration of phosphoric acid in 3RCM (*i.e.*, 0, 0.06, 0.12, 0.36, 1.2, 6, and 12 mM) generated the highest fresh-weight (3.76 ± 0.34) and dry-weight (0.43 ± 0.04) at 6 mM NaH_2PO_4. Saikosaponin-a, -c, and -d contents were unchanged, with production leveling at 14.93, 2.94, and 4.84 mg/L, respectively for 1.2 mM KH_2PO_4 (total: 22.71 mg/L), and 14.24, 2.96, and 5.57 mg/L, respectively at 6 mM (total: 22.77 mg/L). These results suggest that high phosphate concentrations in the culture media are necessary for rapid growth and saikosaponin synthesis, which explains why MS (KH_2PO_4, 1.25 mM) and 3RCM media (NaH_2PO_4, 0.54 mM), containing high phosphate levels, were good at promoting growth in comparison to RCM media, which provides phosphate at a much lower concentration (NaH_2PO_4, 0.18 mM).

Potassium is necessary for the photophosphorylation of chlorophyll and the synthesis and transfer of carbohydrates and protein. Potassium also promotes root strength and growth, which is effective against root rot. However, Fulcheri and co-workers [38] reported that a 5- to 6-fold decrease of potassium concentration in the media stimulated growth and saponin accumulation in *Saponaria officinalis* cell suspension and in *Gypsophila paniculata* root cultures. A similar observation was recorded for the production of solamargine in multiple-shoot cultures of *Solonum paludosum* by controlling potassium concentration [39]. To determine the appropriate media potassium concentration, 3RCM media was adjusted to 0, 0.5, 0.87, 2.6, 8.7, 43.6, and 87.2 mM KCl. Under these conditions, fresh weight (2.85 ± 0.29 mg/L) and dry weight (0.39 ± 0.02 mg/L) were highest at 8.7 mM KCl, with no significant difference to other tested KCl concentrations. Concentrations exceeding 43.6 mM had no further effect on growth. The highest saikosaponin-a, -c, and -d content was measured to be 3.40 ± 0.49, 1.57 ± 0.70, and 2.24 ± 0.11% (total: 7.21%), respectively, at 0.5 mM KCl. The highest saikosaponin accumulation was 25.96 mg/L (saikosaponin-a, 12.24 mg/L; saikosaponin-c, 5.64 mg/L; saikosaponin-d, 8.08 mg/L), which was achieved for 0.5 mM KCl, approximately half the K^+ concentration of regular 3RCM media (0.87 mM) (Fig. 4).

These results indicate that the above four factors considerably affect the growth rate, saikosaponin content, and, ultimately, saikosaponin yield in *B. falcatum* hairy root culture. The sucrose concentration was particularly important to the control of the dry-weight/fresh-weight ratio as well as the saikosaponin yield. Changes in the nitrogen concentration had no appreciable influence on growth, but they did affect the saikosaponin content. Phosphate at moderately high concentrations in culture media improved both root growth and saikosaponin yield. Potassium, on the other hand, could be overdosed, resulting in decreased saikosaponin yield.

It has been shown that the saikosaponin content of field-grown plant roots is related to habitat (total saikosaponin-a and -d content: 0.6~2.6%; average, 1.2%) and plant line (0.6~2.3%; average, 1.2%) [40]. Kusakari *et al.* [26] have reported saikosaponin yields in excess of 2.5% with two-step *B. falcatum* untransformed root culture, where the sucrose concentration was optimized. The saikosaponin content in the present study was higher than 4%, which supersedes the average saikosaponin content of field-grown plants, callus (not detected), untransformed roots (approx. 2.5%), and untransformed root culture treated with methyl jasmonate and $CaCl_2$ (max. 3.1%) [23,26,41].

We believe there is still room for optimization of our culture conditions. The next step will be to examine the impact of other, minor ingredients of 3RCM and MS media, such as $FeCl_3$, Na-EDTA, $MgSO_4$. It will be also worthwhile to look at the effects of some elicitors, biotic (*e.g.*, methyl jasmonate, yeast extract, and chitosan) as well as abiotic (*e.g.*, cadmium, copper, *etc.*) on saikosaponin biosynthesis. The combined data from these studies will ultimately reveal the optimal conditions required for efficient and economical saikosaponin production from *B. falcatum* hairy root cultures.

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