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Baicalin Production in Transformed Hairy Root Clones of *Scutellaria baicalensis*

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Abstract A transformed hairy root clone of *Scutellaria baicalensis* was established following infection with *Agrobacterium rhizogenes* ATCC15834. Three root clones of *S baicalensis* were selected by growth habit and baicalin content. The most active strain—the SR-03 clone—was examined for its growth and baicalin content under various culture conditions. The root growth and baicalin content were maximized in a Schenk and Hildebrandt medium supplemented with 4 and 6% sucrose, respectively. The accumulation of baicalin in transformed hairy roots was enhanced through exposure to various elicitors. Elicitation was attained by the addition of methyl jasmonate, salicylic acid, and various concentrations of fungal cell wall elicitors to the medium. The accumulation of baicalin in the elicited cultures ranged from 10.5 to 18.3 mg/g dry weight of the roots, which was 1.5- to 3-fold the amount attained in controls.

Keywords: baicalin, elicitation, transformed hairy roots, *Scutellaria baicalensis*

INTRODUCTION

Scutellaria baicalensis belongs to the family Labiatae and is a medicinally important plant. This perennial herb has long been used in allopathic and traditional medicine, mainly in Korea, China, Japan, Mongolia, and Russia, *i.e.*, in areas of limited plant growth [1]. In the natural biocenoses, this plant propagates only by seeds and comes to flower only in the tenth year of its life. The root of *S. baicalensis* contains a variety of flavones, phenylethanoids, amino acids, sterols, and essential oils and has been used in China as an important crude drug (known as "wogon" in Japanese) to treat individuals with hepatitis, cancer, diarrhea, and inflammatory diseases [2]. This plant is particularly rich in flavonoid derivatives, among which baicalin, baicalein, wogonin, and wogonin-7-*O*-glucuronide are regarded as active principles. Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone) has been used as an antiinflammatory and antiallergic agent in the treatment of individuals with a variety of inflammatory diseases, such as bronchitis, nephritis, hepatitis, asthma, and atopic dermatitis [3,4]. Plant cells and transformed root cultures are promising potential alternative sources for the production of useful secondary metabolites. Suspension cell cultures have a tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites. Transgenic hairy roots have been explored intensively for their ability to induce a stable and high rate of production of secondary metabolites. Such a transformation of plant cells represents a good strategy for the *in vitro* production of secondary metabolites [5]. Hairy roots obtained through the transformation of higher plants using *Agrobacterium rhizogenes* produced various amounts of useful metabolites, and in most cases more than is produced in normal field-grown plants. The *rolA, rolB,* and *rolC* genes could play a major role in hairy root induction and metabolite production that could also lead to increased production of secondary products in *Atropa belladonna* hairy roots, as is the case with tobacco root cultures [6,7]. Although the possibility of enhancing the accumulation of secondary products by adding elicitors to the medium has been studied extensively in suspension cell cultures, there are a few reports of elicitors being used to transform hairy root cultures. In addition, there are a few reports that hairy roots have been induced from *S. baicalenesis*, and hairy root cultures have been used to produce flavonoids [1,8]. In this report, we describe a study of the effect of chemical culture conditions and elicitors on the production of valuable flavonoids in transformed hairy root cultures of *S. baicalensis*.

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MATERIALS AND METHODS

Plant Materials

S. baicalensis seeds (Richter Herbs, Ontario, Canada) were sterilized by immersion in 70% (v/v) ethanol for 5 min then soaked in a 5% (v/v) sodium hypochloride solution for 10 min and rinsed 3 times in sterile distilled water. Surface-disinfected seeds were transferred to a Murashige and Skoog (MS) medium [9], solidified with 8 g/L agar. The cultures were incubated at 25 ± 1°C for 16

h under a cool fluorescent light (40 μmol/m^2·s^{-1}). Leaves that developed on *in vitro* germinated plantlets were used for the transformation studies, which are described below.

Bacterial Strain

A. rhizogenes ATCC15834 was used for explant inoculation. For transformation experiments, 48 h-old bacteria grown in potato extract medium [10] at 27 ± 1°C were used.

Hairy Root Cultures and Biomass Measurements

To induce root transformation, we took leaves from seedlings grown *in vitro*, cut them into small pieces, and cocultivated them with *A. rhizogenes* ATCC15834. We then excised the induced adventitious roots and cultured them in a half-strength MS (1/2 MS) medium supplemented with cefotaxime 300 mg/L to limit bacterial growth. Three clones of *S. baicalensis* hairy roots obtained after the excision of single root were maintained in a basal MS medium and placed on a shaker set at 100 rpm in the dark. To analyze root growth, we transferred 100 mg (fresh weight) of actively growing roots to 100 mL Erlenmeyer flasks containing 50 mL of half-strength MS, full-strength MS, woody plant medium (WPM), or Schenk and Hildebrandt (SH) medium [11,12] with 3% sucrose. The effect of an initial sucrose concentration of 2, 4, 6, or 8% was also examined. Roots were harvested after 6 weeks in culture, then blotted on filter paper and freeze-dried in an Elya freeze-dryer (EYLA, Tokyo, Japan) before their dry weight was determined.

Treatment of Elicitors

Hairy root cultures of *S. baicalensis* grown in 50 mL of SH medium in 100-mL Erlenmeyer flasks were elicited with fungal cell wall preparations methyl jasmonate (MeJA), and salicylic acid (SA). Fungal cell wall extracts from *Phytophthora megasperma* were used as elicitors according to a method described by McKinley *et al.* [13]. These were administered in various volumes (1∼7 mL) into media-containing flasks. MeJA and SA solutions were prepared in ethanol and added individually to flasks containing 2 week-old roots to final concentrations of 1, 10, and 100 mM, respectively.

Flavonoid Analysis

Flavonoids were determined by a method described by Yu *et al.* [14]. Briefly, hairy root cultures of *S. baicalensis* were freeze-dried for 48 h, ground into a fine powder, and transferred to an amber-colored 20 mL vial. Bioactive compounds were extracted using 25 mL of 50% ethanol sonicated for 10 min and centrifuged at 3,000 rpm for 10 min. The supernatant was filtered through a 4.5 μm nylon syringe filter. The metabolites were separated by high-performance liquate chromatography on a μ-Bondapak C18 column (5% phosphoric acid-acetonitrile, 73∼27 v/v; flow rate: 1.3 mL/min). Baicalin was quantified using an ultraviolet detection device (277 nm) by comparing the findings in this study with authentic standards for baicalin (Wako, Japan) dissolved in 100% ethanol.

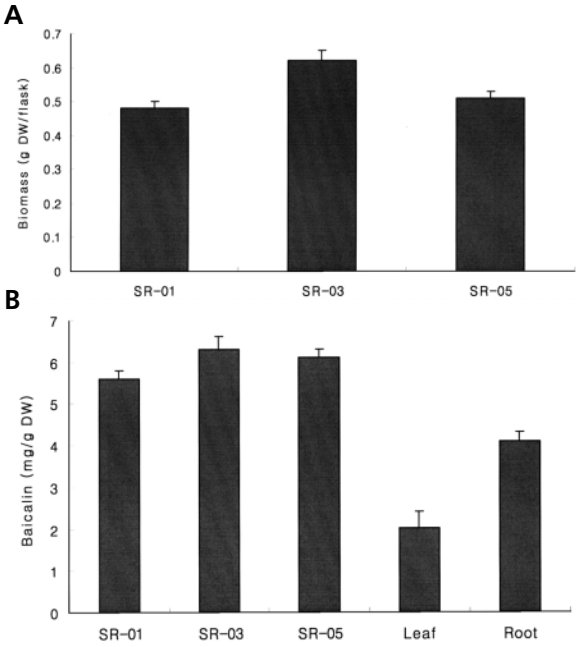

Fig. 1. Growth (A) and baicalin content (B) in hairy root clones of *S. baicalensis.*

RESULTS AND DISCUSSION

Transgenic hairy root cultures of *S. baicalensis* were established through transformation with *A. rhizogenes* ATCC15834. The roots exhibited a very high rate of branching and growth compared with normal cultured roots (data not shown). In general, the hairy root clones were characterized by different morphological categories, their rate of growth (slow, moderate, or fast), and their branching patterns (profusely branched or callused) [5]. We selected 3 adventitious root clones (SR-01, 03, 05) of *S. baicalenesis* based on the phenotypic characteristics of their roots on a solid medium. The SR-03 clone showed the most active growth and baicalin production in a basal medium containing 3% sucrose (Fig. 1A). The baicalin content of the SR-3 clone was also 1.5 to 3.0 times greater than that in the leaf and root parts of cultured plantlets *in vitro* (Fig. 1B). Thereafter, the SR-03 clone was used to determine optimal culture conditions.

The Effect of Basal Media on the Growth of Hairy Roots and the Production of Baicalin in Roots

Secondary metabolite biosynthesis in transformed roots is genetically controlled, but it is influenced by nutritional and environmental factors. Therefore, the composition of the culture medium can affect growth and

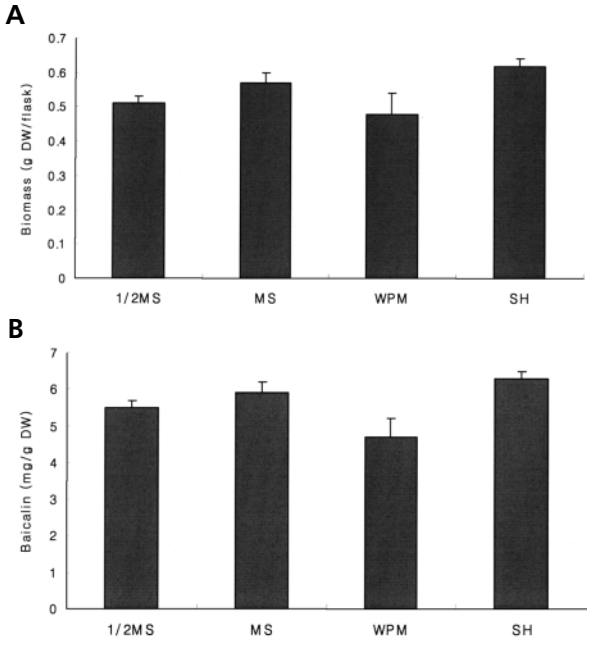

Fig. 2. Effect of basal media on growth (A) and baicalin content (B) in a hairy root clone of *S. baicalensis.*

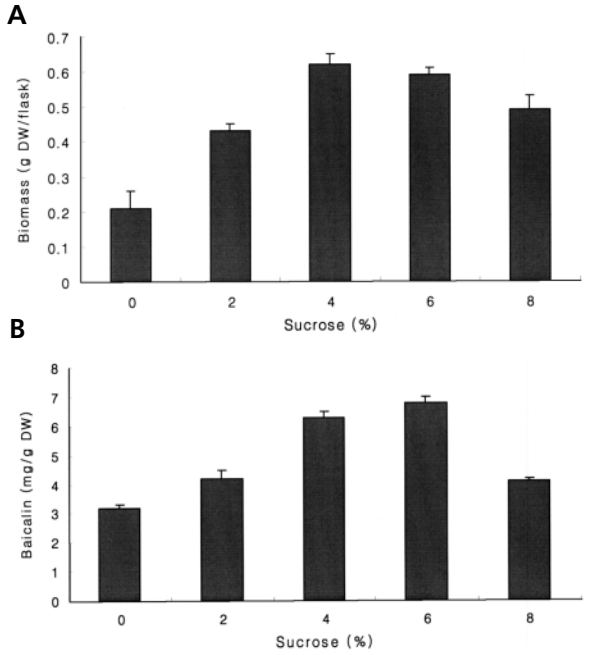

Fig. 3. Effect of initial sucrose concentration on growth (A) and baicalin content (B) in a hairy root clone of *S. baicalensis.*

secondary metabolite production [15,16]. Of the 4 basal media tested, the SH medium supported the most growth and the highest baicalin content in transformed the hairy root clone (SR-03) of *S. baicalenesis* (Fig. 2). Neither the WPM nor the half-strength MS media were found to be suitable for growing the *S. baicalenesis* hairy root. Dilution of the culture media and a reduction in the nitrogen concentration enhance growth and the alkaloid content of hairy root cultures in the Solanaceae family [17,18]. Merkli *et al.* [19] reported that in *Trigonella foenum-graecum* hairy root cultures, the fastest growth was seen in the WPM medium and the highest diosgenin content was observed in the half-strength WPM medium. On the other hand, Ikenaga *et al.* [20] achieved optimal growth and steroid saponin production in *Solanum aculeatissimum* hairy root cultures using a B5 medium containing 3% sucrose.

Effect of Sucrose Concentration on the Growth of Hairy Roots and the Production of Baicalin

Previous tissue culture studies have indicated that the initial sucrose concentration can affect a range of culture parameters, such as growth rate and yield of secondary products, as well as the ratio of fresh weight to dry weight [21]. The optimum concentration of sucrose required for growth and baicalin content production in *S. baicalensis* hairy root cultures was evaluated in the SH medium with a sucrose concentration ranging from 2 to 8%. The biomass and baicalin content after 6 weeks in culture are shown in Fig. 3. The growth of hairy roots was the highest in media containing 4% sucrose compared with 2% sucrose (the amount of biomass produced was higher by about 0.62 g dry weight per flask); the baicalin content accumulated to a maximum of 6.3 mg/g DW with an initial sucrose concentration of 6%. A similar phenomenon was reported in other cell cultures. For example, Zhong *et al.* [22], in examining the effect of initial sucrose concentrations, reported that maximum root growth and saponin accumulation were obtained with 4 and 6% sucrose, respectively. Indole alkaloid accumulation was found to be optimal in cell cultures of *Catharanthus roseus* with an 8% sucrose concentration compared with concentrations in the range of 4 to 12% [23]. However, Sakamoto *et al.* [24] reported that a higher concentration of sucrose (5%) reduced anthocyanin production in cultures of *Aralia cordata* cells, while a lower concentration (3%) enhanced the accumulation of anthocyanin.

The Effect of Elicitors on the Growth of Hairy Roots and the Production of Baicalin

Elicitation has a negative effect on growth. Manthe *et al.* [25] reported a 25% reduction in the growth of *Vicia faba* after 7 days of treatment with 5-mM SA. When transformed belladonna roots were treated with 2 mM SA for 24 h, the subcultured biomass on day 14 was reduced to 9% of controls. In our experiment, neither MeJA nor SA had a highly negative effect on growth in hairy roots (data not shown). By contrast, they each stimulated baicalin production in *S. baicalensis* hairy roots. It has been reported that MeJA and SA are endogenous signal molecules that not only elicit plant resistance to pathogens and herbivores but also exogenously induce secondary metabolic pathway activity [26]. Fig. 4 shows the rate of production for baicalin over 6 weeks in the presence of

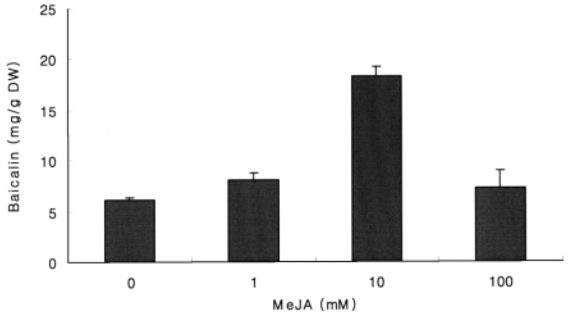

Fig. 4. Effect of MeJA on baicalin content of a hairy root clone of *S. baicalensis*.

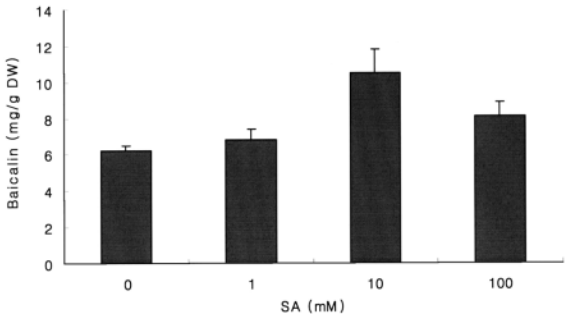

Fig. 5. Effect of SA on baicalin content in a hairy root clone of *S. baicalensis*.

various concentrations of MeJA. After exposure to 10 mM MeJA, the baicalin content of the roots increased initially and slowly decreased thereafter, reaching a maximum yield of 18.3 mg/g dry weight. Ketchum *et al.* [27] observed an optimal MeJA concentration of 200 μM for taxol production in *Taxus canadensis* cell cultures. Bulgakov *et al.* [28] reported that 10 μM MeJA strongly increased the accumulation of anthraquinones in transformed callus cultures of *Rubia cordifolia*. Additionally, various concentrations of SA improved baicalin production compared with controls (Fig. 5). At an SA concentration of 10 mM, the baicalin content reached a maximum of 10.5 mg/g dry weight. SA and its chemical derivative have been reported to enhance the productivity of some metabolites in plant tissue and cell cultures. In a suspension culture of *Hyosycamus muticus* treated with 40 μM SA, for example, lubimin production increased by 50%, while in a transformed root culture of the same species, solavetivone production increased by 48% following the addition of 4 μM SA [29]. Furthermore, an increase in total alkaloid production of 505% was achieved by adding 1 mM ASA to *Catharanthus roseus* tumor lines *in vitro* [30]. An elicitor extracted from *P. megasperma* was also tested for its effect on baicalin accumulation in hairy root cultures of *S. baicalenesis* (Fig. 6). Elicited root cultures presented much more accumulation of baicalin than controls. Yuan *et al.* [31] reported that the addition of a fungal elicitor enhanced the taxol yield in *Taxus chinensis* cell cultures. These results are similar to those reported in other studies of the effect of

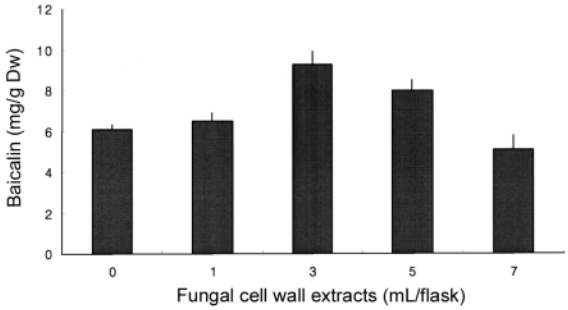

Fig. 6. Effect of fungal cell wall extracts on baicalin content in a hairy root clone of *S. baicalensis*.

fungal elicitation on various plants [32-34]. In the case of MeJA, elicitor exposure produced a higher rate of baicalin production than SA or fungal cell-wall elicitors.

CONCLUSION

Hairy roots of *S. baicalenesis* were obtained following the co-culture of its seedlings with *A. rhizogenes* ATCC15834. Three root lines were established and examined for rates of growth and baicalin productivity under various culture conditions. The optimal rate of root accumulation occurred on hormone-free SH medium containing 4% sucrose. The baicalin content of *S. baicalenesis* hairy root cultures was enhanced approximately 1.5- to 3.0-fold through the addition of elicitors to the basal medium.

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