

Sennosides A and B Production by Hairy Roots of *Senna alata* (L.) Roxb.

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Hairy roots of *Senna alata* transformed with *Agrobacterium rhizogenes*, strain ATCC 15834 were induced and grown in half-strength Murashige and Skoog (MS) medium. Effects of sucrose contents and hormones on the growth and sennosides A, B production were investigated. Hairy roots cultured on hormone-free half-strength MS medium containing 5% sucrose under dark condition mostly stimulated the growth of hairy roots and increased the content of sennosides A and B yielding (169 ± 4) and (34 ± 3) $\mu\text{g g}^{-1}$ dry wt, respectively.

Key words: Sennosides A and B, Hairy Roots

Introduction

Senna alata (L.) Roxb. (family Leguminosae) is known to contain anthraquinone glycosides such as sennosides A and B, which act as laxative (Elujoba *et al.*, 1989). *S. alata* has been reported to have many pharmacological activities such as antimicrobial (Somchit *et al.*, 2003), laxative (Elujoba *et al.*, 1989), and analgesic activity (Palanichamy and Nagarajan, 1990). Sennosides are still manufactured as the pharmaceutical product from the plant extracts.

Anthraquinone production from the tissue culture has been optimized for individual species (Nazif *et al.*, 2000; Sepehr and Ghorbani, 2002; Ahuja and Sambyal, 1991). The accumulation of anthraquinone derivatives like chrysophanol and emodin was obtained from the tissue culture of *Cassia tora* (Tabata *et al.*, 1975), *C. acutifolia* (Nazif *et al.*, 2000), *C. fistula* (Ahuja and Sambyal, 1991) and *C. obtusifolia* (Takahashi *et al.*, 1978). On the other hand, a few investigations concerning sennosides A and B formation by tissue culture were reported (Ohshima *et al.*, 1988). Recently, hairy root cultures were useful for the production and biosynthesis of plant secondary metabolites in many plant species because of biochemical stability and rapid growth rate (Lorence *et al.*, 2004; Sasaki *et al.*, 1998; Weathers *et al.*,

1994). It has been reported that hairy roots of *C. obtusifolia* contain free anthraquinones; aloemodin, chrysophanol, 8-*O*-methyl-chrysophanol, rhein and emodin (Guo *et al.*, 1998). However, hairy roots of *S. alata* and their potential use for production of sennosides A and B have not been reported. Therefore, we are interested in determining sennosides A and B from the established hairy roots of *S. alata*. In addition, the effect of culture conditions for the formation of sennosides A and B has been investigated in our laboratory.

Experimental

Chemicals and immunochemicals

Human serum albumin (HSA) was provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (Durham, NC, USA). Sennoside A and sennoside B were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other chemicals were standard commercial products of analytical grade.

Plant materials

S. alata seeds were sterilized with 10% (v/v) sodium hypochlorite for 20 min. After rinsing with sterile distilled water, seeds were aseptically germinated on hormone-free Murashige and Skoog

(MS) medium supplemented with 3% (w/v) sucrose at $(25 \pm 2)^\circ\text{C}$ under a 16 h photoperiod with fluorescent light.

Agrobacterium rhizogenes-mediated transformation

The seedlings (21-day-old) were used as explant for transformation. *Agrobacterium rhizogenes* strain ATCC 15834 was grown in YEB liquid medium at 28°C overnight using a rotary shaker. The seedling plants were inoculated with *A. rhizogenes* by wounding on stem and leaves regions of plant with a needle previously dipped into the bacterial suspension. The hairy roots were induced from the wound site on the plant after inoculation for 2 weeks. The roots were cultured on hormone-free half-strength MS medium (3% w/v sucrose) supplemented with $500\ \mu\text{g ml}^{-1}$ cefotaxime at 25°C under a 16 h photoperiod with fluorescent light. After three passages onto new antibiotic-containing medium at 14-d intervals, the bacteria-free hairy roots were transferred into hormone-free half-strength MS liquid medium. The hairy roots were subcultured every 3 weeks into fresh medium.

Total DNA from hairy roots and non-transformed roots were extracted using established method (Edwards *et al.*, 1991). Polymerase chain reaction (PCR) identification of the rooting locus gene *rolB* was performed. The primer used to amplify the *rolB* gene (780-bp) on the T-DNA of *A. rhizogenes* ATCC 15834 Ri plasmid was designed according to Hamill *et al.* (1991). The primer designed to amplify *rolB* was 5'-ATGGATCCCAAATTGCTATTCCTTCCA CGA-3' and the reverse primer was 5'-TTAGGCTTCTTTCTTCA GGTTCAC TGCAGC-3'. Each PCR reaction was carried out on 20–30 ng of total DNA with 1.2 units *Tag*DNA polymerase (Pharmacia) in a volume of $30\ \mu\text{l}$ (Hamill *et al.*, 1991). For amplification, the PCR parameters consisted of denaturing at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 3 min, with 30 cycles. The amplified samples were analyzed by electrophoresis on a 1% agarose gel and detected by staining with ethidium bromide.

Growth rates, effects of sucrose and hormones on the production of sennosides A and B

The hairy root cultures were maintained in 125-ml Erlenmeyer flasks containing 30 ml of the

liquid half-strength MS medium (3% w/v sucrose), grown under a 16 h photoperiod at 25°C with agitation on a rotary shaker (100 rpm) and harvested every 5 d for time-course study. Various contents amounts of sucrose and hormones were added to the liquid culture medium to test their effect on the growth of hairy roots and sennosides A and B production under light (16 h photoperiod) or dark condition at 25°C with agitation (100 rpm). After 30-d culture, the dry weight and sennosides A and B contents were determined. Each experiment was done in triplicate.

Sample preparation

Hairy roots (30 mg dry wt) were extracted four times with $500\ \mu\text{l}$ methanol containing 0.1% NH_4OH using an ultrasonic bath for 15 min. After filtration and evaporation to dryness, the residue was resuspended in 1 ml of 10 mM NaHCO_3 . Sennosides A and B contents were then determined by competitive ELISA using an anti-sennoside A monoclonal antibody and an anti-sennoside B monoclonal antibody.

Determination of sennosides A and B by competitive ELISA

Sennosides A and B in sample solutions were determined by a competitive ELISA as previously reported with a modification (Morinaga *et al.*, 2000, 2001). Sennoside A-HSA ($100\ \mu\text{l}$ of $1\ \mu\text{g ml}^{-1}$) was adsorbed onto the wells of a 96-well immunoplate and then treated with $300\ \mu\text{l}$ phosphate buffered saline (PBS) containing 5% skim milk (S-PBS) for 1 h. $50\ \mu\text{l}$ of various concentrations of sennoside A or samples diluted in 10 mM NaHCO_3 solution were incubated with $50\ \mu\text{l}$ of monoclonal antibody solution for 1 h. The plate was washed three times with PBS containing 0.05% Tween 20 (T-PBS), and then incubated with $100\ \mu\text{l}$ of a 1,000-fold dilution of peroxidase-labeled goat anti-mouse IgG for 1 h. After washing the plate with T-PBS, $100\ \mu\text{l}$ of substrate solution [0.1 M citrate buffer, pH 4.0, containing 0.003% H_2O_2 and $0.3\ \text{mg ml}^{-1}$ 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] were added to each well and incubated for 15 min. The absorbance was measured by a microplate reader (Model 550 Microplate Reader Bio-Rad Laboratories, Hercules, CA) at 405 nm. All reactions were carried out at 37°C . The sennoside B content was determined in the same manner.

Results and Discussion

Yellowish hairy roots of *S. alata* were generated from the wounded sites after 2 weeks of inoculation on half-strength MS medium containing $500 \mu\text{g ml}^{-1}$ cefotaxime. PCR was performed to

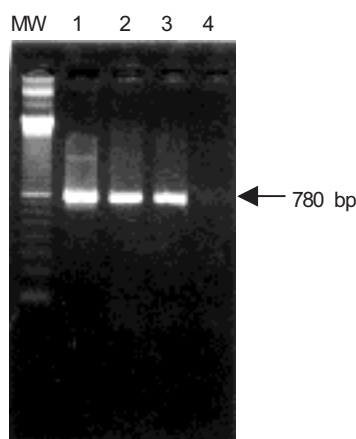


Fig. 1. Agarose gel electrophoresis of PCR amplified DNA fragment of *ro/B* gene. Lanes: MW, 100-bp ladder; 1, DNA from *A. rhizogenes* ATCC 15834 (positive control); 2, hairy roots clone #1; 3, hairy roots clone #3; 4, non-transformed roots. The expected PCR product (780 bp) is indicated with an arrow.

confirm the integration of the *ro/B* gene in hairy roots induced by *A. rhizogenes* ATCC 15834 (Fig. 1). The *ro/B* gene specific primer amplified a 780-bp fragment from *A. rhizogenes* and DNA extracted from transformed roots of *S. alata*. The amplified fragment from hairy root clones yielded the expected band for these primers and no band was obtained from non-transformed roots. Hairy root clones of *S. alata* transformed with *A. rhizogenes* displayed a similar morphological characteristic of different clones. We chose clone #3 which has the greatest biomass production for further experiments. The time course study of the growth of hairy roots was carried out by using the shaking culture, and sennosides A and B contents were quantified simultaneously by ELISA using anti-sennoside A and sennoside B monoclonal antibodies as shown in Fig. 2. The rapid growth of hairy roots exhibited a growth curve from day 5 to day 20 on which the highest root weight was observed (Fig. 2). Sennosides A and B contents in hairy roots decreased during 10 days due to the growth of hairy roots. After this lag phase, production of sennosides A and B showed an increase from day 15 and reached its maximum level in the stationary phase of hairy roots by day 35 $[(178 \pm 15) \text{ and } (23 \pm 2) \mu\text{g g}^{-1} \text{ dry wt, respec-}]$

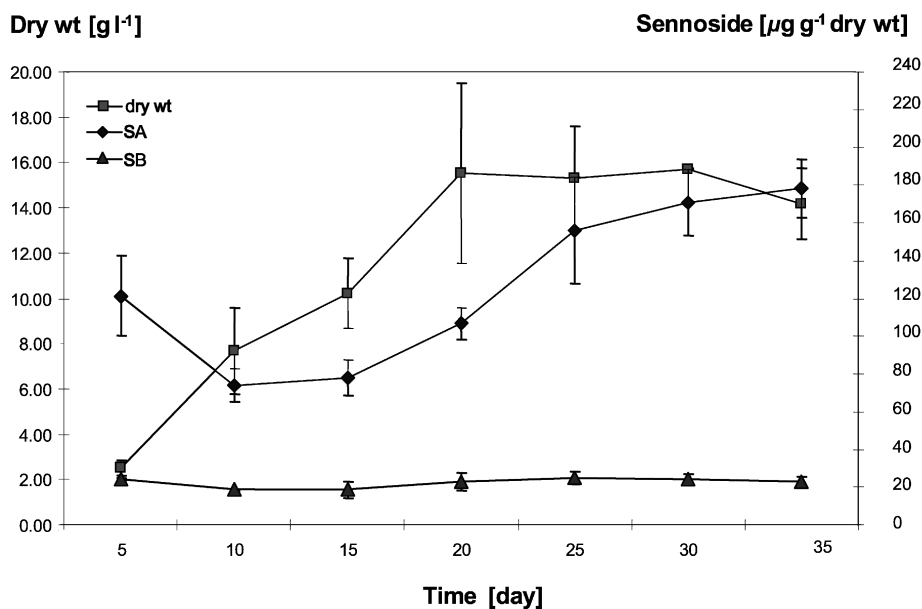


Fig. 2. Time-course for growth and sennosides A and B content in *S. alata* hairy roots. Hairy roots were determined in half-strength MS liquid medium with agitation (100 rpm). The values are means of triplicate results and error bars show standard deviations.

tively]. This evidence suggests that the biosynthesis of secondary metabolite increased during the stationary phase of growth. Similar observations have been noted for other hairy root cultures (Bhadra and Shanks, 1997; Merkli *et al.*, 1997; Sasaki *et al.*, 1998; Kittipongpatana *et al.*, 2002). Increased production of secondary metabolite at the stationary phase might be come from the stress of tissue due to the change of environment such as undernourishment. In addition, during 35 days of culture, no sennosides A and B were detected in the culture medium suggesting that sennosides A and B were retained within the tissues. *S. alata* hairy roots showed a stable and fast growth rate, and produced sennosides A and B steadily. The production of sennosides A and B in non-transformed roots was found to be (181 ± 7) and $(22 \pm 2) \mu\text{g g}^{-1}$ dry wt, respectively. The amounts of sennosides A and B produced in hairy roots were not significantly different from those produced in non-transformed roots when studied at the same cultured period.

The initial concentration of sugar in the medium affects the cell growth and yield of secondary metabolite in many cases (Lui *et al.*, 1997; Kittipongpatana *et al.*, 1998; Nishiyama and Yamakawa, 2004). We tested the influence of sucrose contents on biomass and sennosides A and B production in hairy roots. The biomass of hairy roots was increased in proportion to an increment of the sucrose content in the medium excluding the high sucrose contents at 8% and 10% (w/v). The suppression of hairy root growth in the medium containing 8% and 10% (w/v) sucrose could be due to the high osmotic pressure at high sucrose contents. The lowest biomass of hairy roots was observed at 1% (w/v) sucrose. The optimal condition of the culture growth of hairy roots was observed for medium containing 5% (w/v) sucrose under dark condition. Sucrose content has effects on sennosides A and B production in hairy root culture of *S. alata*. High sennosides A and B contents were achieved in medium containing 3% and 5% (w/v) sucrose, and higher contents of sucrose reduced sennosides A and B production (Fig. 3). Sennosides A and B contents in hairy roots reached (169 ± 4) and $(34 \pm 3) \mu\text{g g}^{-1}$ dry wt, respectively, in the medium containing 5% (w/v) sucrose (Fig. 3). These levels of sennosides A and B were higher than those in callus of *Rheum* spp. (83 and $4 \mu\text{g g}^{-1}$ dry wt, respectively) (Ohshima *et al.*, 1988). In addition, it became evident that under

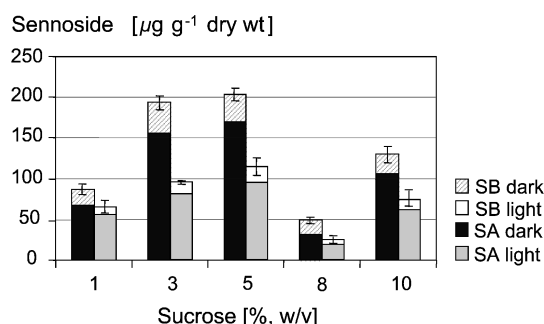


Fig. 3. Influence of sucrose content on sennosides A and B content in *S. alata* hairy roots. The values are means of triplicate results and error bars show standard deviations.

dark condition higher contents of sennosides A and B were produced than light condition.

We studied the influence of plant growth hormones on the sennosides A and B production using liquid half-strength MS medium containing hormones (2,4-D, NAA, BA or kinetin) at concentration 1 mg l^{-1} . The control culture under dark condition had the highest level of sennosides A and B namely (155 ± 8) and $(38 \pm 0) \mu\text{g g}^{-1}$ dry wt, respectively (Fig. 4). Conversely, the production of sennosides A and B was inhibited when cultured in medium containing hormones. The addition of 2,4-D to the medium strongly decreased the sennosides A and B contents (93%). It is evidence that 2,4-D at 1 mg l^{-1} strongly suppressed growth and sennosides A and B production. These results show that hormones added to the medium have attributed the negative effects on sennosides A and B production of *S. alata* hairy roots. Fur-

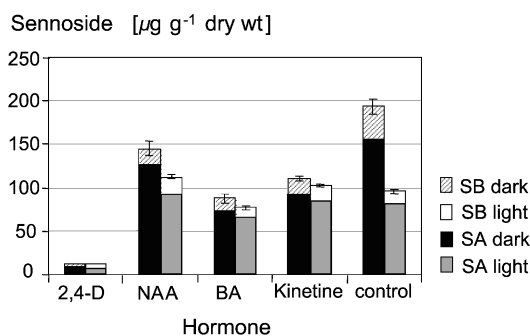


Fig. 4. Influence of hormones on sennosides A and B content in *S. alata* hairy roots. The values are means of triplicate results and error bars show standard deviations.

thermore, the hairy root cultures containing hormones under dark condition accumulated higher sennosides A and B contents than that under light condition. Therefore, we concluded that the hairy roots cultured under dark condition were suitable for the production of sennosides A and B.

Overall, growing hairy root cultures of *S. alata* on hormone-free half-strength MS medium containing 5% (w/v) sucrose under dark condition is the optimum condition for biomass formation and producing sennosides A and B by the shaking culture system. The hairy roots culturing presented herein may be a promising tool for a biosynthetic

study of sennosides A and B. Therefore, the studies of biosynthesis precursors and effects of elicitor enhancing sennosides A and B production in *S. alata* hairy roots are now in progress.

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