Dicentrine Production from a Hairy Roots Culture of *Stephania suberosa*

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A hairy roots culture of *Stephania suberosa* was established using *Agrobacterium rhizogenes* ATCC15834. The production of dicentrine was found to be (8.92 ± 0.07) mg/g dry wt on day 35 of culture. Effects of sucrose content, tyrosine, and medium strength on growth and dicentrine production of *S. suberosa* were investigated. 6% (w/v) sucrose was an optimum content for the growth and dicentrine accumulation in *S. suberosa* hairy roots. The utilization of a precursor from tyrosine feeding enhanced the dicentrine production. The medium with 1.0 mM of tyrosine had the highest effect on dicentrine accumulation in hairy roots at day 40 of culture [(14.73 ± 0.47) mg/g dry wt]. In addition, ¼ Murashige and Skoog medium was suitable for biomass and dicentrine production in hairy roots. This culture system has a potential to produce dicentrine from hairy roots of *S. suberosa*.

Key words: Dicentrine, Hairy Roots, *Stephania suberosa*

Introduction

Stephania suberosa Forman (Menispermaceae) is a medicinal plant which has been used in Thai traditional medicine for the treatment of hypertension. The chemical constituents isolated from *S. suberosa* tuberous roots are aporphine alkaloids, like dicentrine and isolaureline (Sriprang *et al*., 2006), and bisbenzylisoquinoline alkaloids, like cepharanthine, norcepharanthine, stephasubine, and norstephasubine (Patra *et al*., 1986). The aporphine alkaloid dicentrine is one of the major active compounds isolated from the plant tuber (Sriprang *et al*., 2006). Dicentrine shows antitumour activity (Stevigny *et al*., 2005; Woo *et al*., 1999), α1-adrenoceptor blocking (Mustafa and Achike, 2000), antiarrhymic action (Young *et al*., 1994), antiplatelet effect (Chen *et al*., 1996), and acetylcholinesterase inhibition (Sriprang *et al*., 2006; Ingkaninan *et al*., 2003). Due to these pharmacological effects, plants producing high amounts of compounds are needed as source of material for the pharmaceutical area. Recently, plant tissue culture techniques became a potential tool for the production of secondary metabolites of medicinal plants. A hairy roots culture is an alternative method to produce secondary metabolites, because of its rapid growth and genetic stability. The accumulation of alkaloids was achieved in *S. suberosa* tuberous roots (Patra *et al*., 1986; Sriprang *et al*., 2006). Therefore, hairy roots cultures of *S. suberosa* are necessary for the production of biological compounds. It is known that many factors could affect the secondary metabolites production in hairy roots cultures including media components, carbon sources, and precursor feeding. It would, therefore, be interesting to establish hairy roots cultures of *S. suberosa* for the production of dicentrine. In the present study, we hereby report on hairy roots cultures of *S. suberosa* investigating the effect of sucrose content, tyrosine feeding, and medium strength on the hairy roots culture for optimizing cell growth and dicentrine production.

Material and Methods

Chemical reagents

Dicentrine (9,10-dimethoxy-1,2-methylenedioxyaporphine) was obtained from Sequoia Research Products Ltd. (Pangbourne, United Kingdom). Cefotaxime (CF) sodium salt was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Tyrosine was obtained from Fluka Chemical (Buchs, Switzerland). All other chemicals were standard commercial products of analytical grade.

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Hairy roots induction

S. suberosa was obtained from Botanical Garden, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. Plants were surface-sterilized in 10% sodium hypochlorite for 15–20 min, washed three times with sterilized water, and then immersed in 70% ethanol for 1 min. Stem segments were infected with *Agrobacterium rhizogenes* ATCC15834 and cultured on Murashige and Skoog (MS) medium at 25 °C for 48 h, and then transferred to half-strength (½) MS medium with 500 mg/l CF. At 2-week-intervals, infected segments were transferred to ½ MS medium with 300 mg/l, 100 mg/l CF, and without CF, respectively. Transformed roots of *S. suberosa* were grown in 125-ml flasks containing 30 ml of ½ MS liquid medium. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). The hairy roots were subculture every 4 weeks into fresh medium.

Growth rate and effects of sucrose and medium strength on the dicentrine production in hairy roots

Fully grown hairy roots were subcultured into 125-ml flasks containing 30 ml of ½ MS liquid medium. Hairy roots were harvested every 5 d to determine the fresh weight (wt), dry weight and dicentrine content. Various contents of sucrose (3–12% w/v) and several medium strengths (2MS, MS, ½ MS, ¼ MS and $^1/_8$ MS) were used to test their effect on the growth of hairy roots and dicentrine production under light (16 h photoperiod) conditions at 25 °C with agitation (100 rpm). After 30 d of culture, the dry weight and dicentrine content were determined. Each experiment was done in triplicate.

Effect of precursor feeding on the dicentrine production in hairy roots

Various contents of tyrosine (0.5, 1.0, 1.5 mM) were added to 125-ml flasks containing 30 ml of ½ MS liquid medium to investigate the growth and dicentrine production. Hairy roots were harvested every 10 d to determine the dry weight and dicentrine content. Each sample was done in triplicate.

Extraction of samples and dicentrine analysis

Dried samples (30 mg) were powdered and extracted four times with 0.5 ml methanol with sonication. The extracts were combined, evaporated and then redissolved in 1 ml methanol. The dicentrine contents were determined by HPLC using a Hewlett Packard series 1100 instrument with a UV/VIS detector (308 nm) and a HP 3396 series

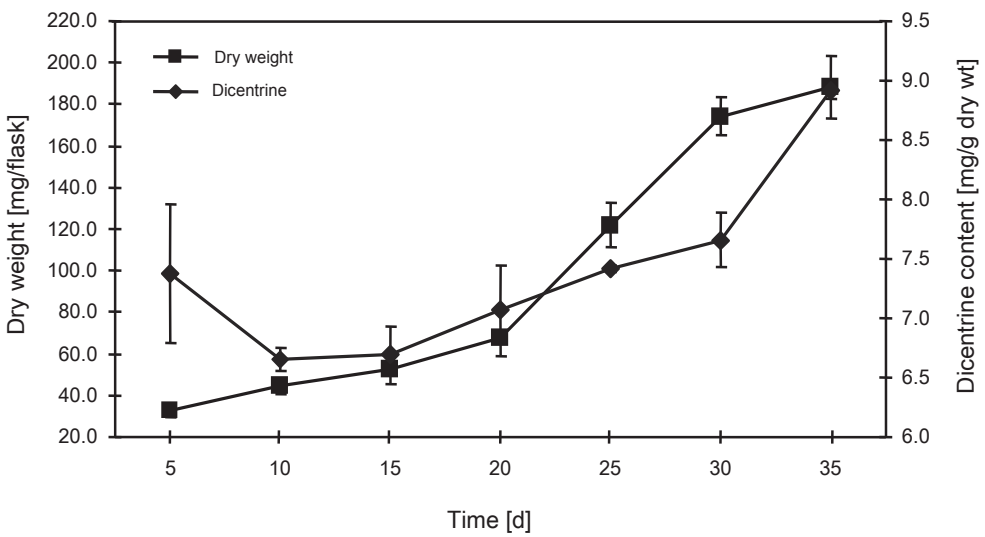

Fig. 1. Time course of growth and dicentrine production of a *S. suberosa* hairy roots culture on liquid ½ MS medium for 35 days.

III integrator. An RP-18 column (LiChroCART®, 125 mm × 4 mm, 5 μm particle size, Merck, Germany) was used. The mobile phase consisted of 40% acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min. Each sample was examined in triplicate.

Results and Discussion

Hairy roots of *S. suberosa* commonly emerged from the wounded sites after 2 weeks of inoculation on a solid culture of ½ MS medium containing 500 mg/l CF. Then, transformed roots were grown in ½ MS liquid medium under light (16 h photoperiod) conditions at 25 °C with agitation (100 rpm). The hairy roots grew slowly in the first 15 days of culture and grew faster subsequently, during days 20–30 (Fig. 1). The dry weight of *S. suberosa* hairy roots increased from (33.00 ± 3.20) mg dry wt/flask to (188.30 ± 15.10) mg dry wt/flask by day 35. Moreover, using HPLC analysis, the dicentrine content in hairy roots increased up to (8.92 ± 0.07) mg/g dry wt by day 35 (Fig. 1). The dicentrine content is thus proportional to the growth rate of hairy roots. The maximum biomass and dicentrine production of the hairy roots culture occurred on day 35 of culture.

½ MS medium containing various sucrose contents was investigated for its effects on biomass and dicentrine production. A sucrose content of 6% (w/v) provided the highest growth rate of *S. suberosa* hairy roots (Fig. 2A). 3% and 6% (w/v) sucrose produced high levels of dicentrine which were found to be (7.61 ± 0.21) mg/g dry wt and (7.53 ± 0.34) mg/g dry wt, respectively (Fig. 2B). This result was similar to those found for hairy root cultures of *Datura quercifolia* concerning the biomass and alkaloid production (Dupraz *et al.*, 1994). Therefore, we concluded that the optimum sucrose content for biomass and dicentrine production is 6% (w/v).

Tyrosine is an amino acid precursor for the production of aporphine alkaloids in the biosynthesis pathway. Therefore, we tested the effects of tyrosine on the growth of hairy roots and the content of dicentrine. The biomass of hairy roots slightly decreased at high tyrosine contents (Fig. 3A). Fig. 3B shows that the dicentrine production increased in proportion to the hairy roots culture period. The use of tyrosine at all concentrations resulted in an increased dicentrine level (Fig. 3B). This finding suggested that the utilization of a precursor from tyrosine feeding can enhance the dicentrine production. These results were similar to those of previous studies on a hairy roots culture of *Rhodiola sachalinensis* (Zhou *et al.*, 2007). Among various contents of tyrosine, the medium with 1.0 mM tyrosine had the highest effect on dicentrine accumulation in hairy roots and increased up to (14.73 ± 0.47) mg/g dry wt by day 40. These results suggested that the optimal concentration for precursor was 1.0 mM tyrosine.

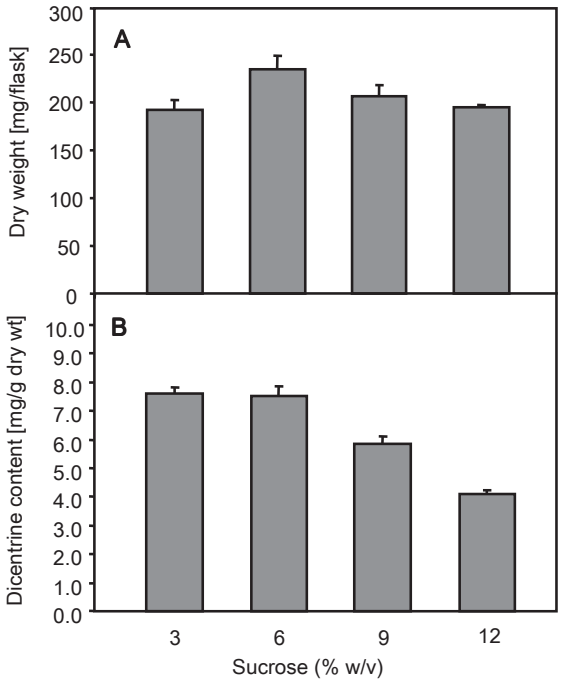

Fig. 2. Effect of sucrose in medium on (A) dry weight and (B) dicentrine production of *S. suberosa* hairy roots.

Medium strength is one of the factors that can affect the root development (Suzuki *et al.*, 1992). We studied the effect of medium strength on biomass and dicentrine production in hairy roots. The biomass and dicentrine production increased at lower strength of the medium (Fig. 4). Fig. 4A shows that ¼ MS medium produced the highest dry weight [(183.80 ± 6.30) mg/flask]. The lowest dry weight of biomass of hairy roots was observed with 2MS medium. The dicentrine accumulation in hairy roots appeared to be closely related to biomass accumulation; ¼ MS medium produced the highest level of dicentrine which was found

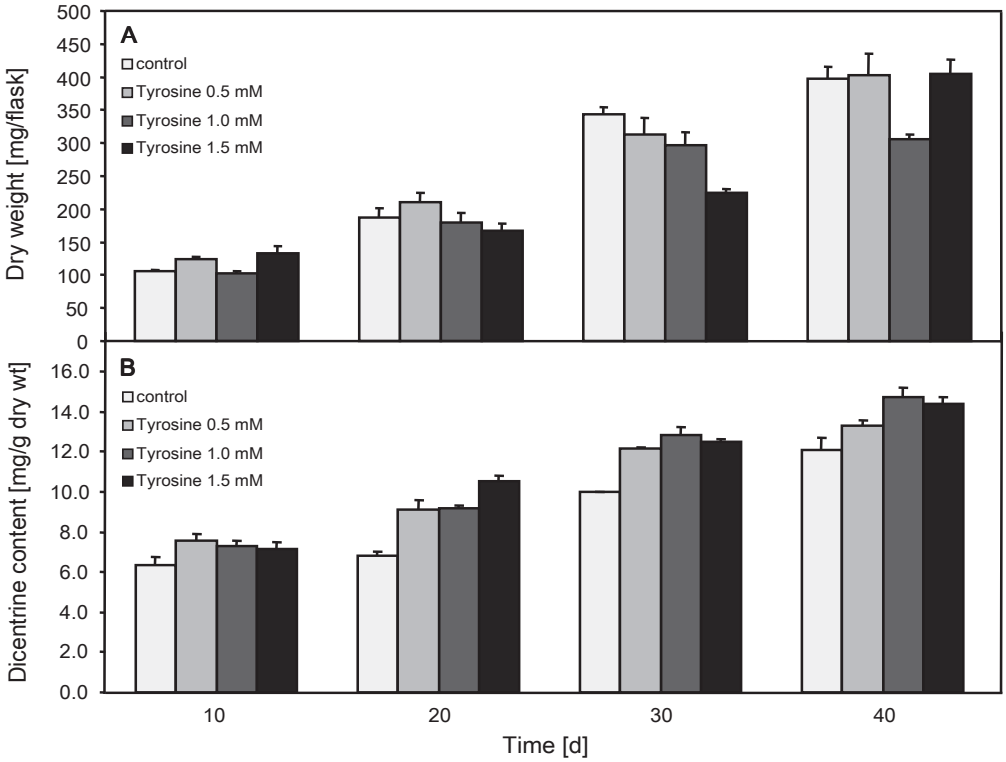

Fig. 3. Effect of tyrosine in medium on (A) dry weight and (B) dicentrine production of *S. suberosa* hairy roots.

to be (10.61 ± 0.32) mg/g dry wt (Fig. 4B). These results indicated that ¼ MS medium had the highest effect on biomass and dicentrine accumulation in hairy roots of *S. suberosa*.

In conclusion, 6% (w/v) sucrose was an optimum content for a hairy roots culture of *S. suberosa*. Addition of 1.0 mM tyrosine to the culture medium had the highest effect on dicentrine accumulation in hairy roots at day 40 of culture. ¼ MS medium was effective for hairy roots culture of *S. suberosa*. Therefore, hairy roots of *S. suberosa* from this study have a potential for the production of dicentrine.

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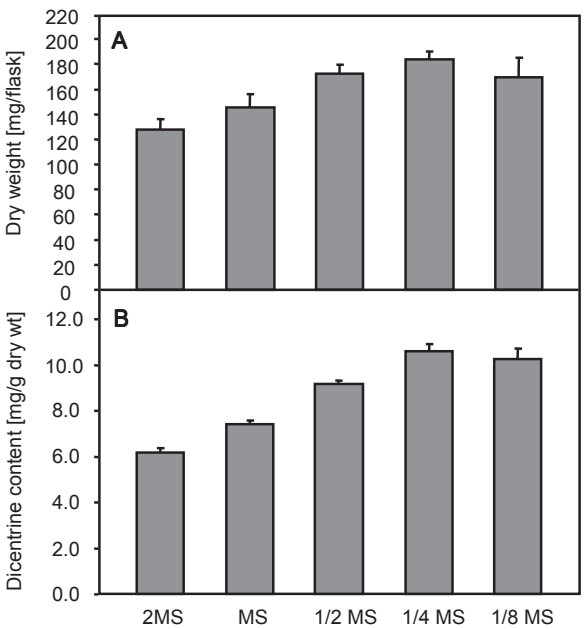

Fig. 4. Effect of medium strength on (A) dry weight and (B) dicentrine production of *S. suberosa* hairy roots.

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