

Adventitious Root Culture and *In Vitro* Production of Dioscin from *Smilax china* L.

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Abstract - An adventitious root formation protocol from *Smilax china* L. was established for *in vitro* production of dioscin, a steroidal saponin having various bioactivities such as anticancer, antifungal, antiviral, and antiobesity. Optimal medium for root initiation from leaf explant was MS medium containing $30 \text{ g} \cdot \text{L}^{-1}$ of sucrose supplemented with $1.0 \text{ mg} \cdot \text{L}^{-1}$ kinetin + $2.0 \text{ mg} \cdot \text{L}^{-1}$ NAA. The induction of adventitious roots from *in vitro* initiated root segments was most favorable to MS liquid medium with $0.1 \text{ mg} \cdot \text{L}^{-1}$ kinetin + $2.0 \text{ mg} \cdot \text{L}^{-1}$ NAA. Among the 20 different adventitious roots originated from different plants, strain No. 10 was selected based on production ability of dioscin, and its stability through the successive suspension culture. The maximum growth stage of adventitious roots was noticed at 5 weeks after subculture while that of dioscin production in the adventitious root was at 7 weeks after subculture in suspension culture system. These results provide that suspension culture of adventitious roots of *Smilax china* L. have a potential for *in vitro* mass production of dioscin.

Key words - Adventitious root, Dioscin, Steroidal saponin, Suspension culture

Introduction

Smilax china L., a perennial plant growing in mountainous areas of the Korean peninsula, has long been used as a folk medicine for asthma, rheumatoid arthritis, and other disease in Korea and China. The pharmaceutically active components of the *Smilax china* L. was identified as dioscin, a steroidal saponin showed various bioactivities such as anticancer activity (Hu *et al.*, 1996; Hu and Yao, 2003; Li *et al.*, 2003; Cai *et al.*, 2003), antimicrobial activity (Vasiukova *et al.*, 1977; Sautour *et al.*, 2004), lipase inhibitory activity (Kwon *et al.*, 2003), and antiviral activity (Aquino *et al.*, 1991). Also, diosgenin and related saponins are well known starting materials for the manufacture of pharmaceutically important steroids, and can be obtained from tubers, seeds, or cultivated cells of various plants (Tal and Goldberg, 1982). Although the chemical synthesis of dioscin and dioscin derivatives was recently succeeded (Deng *et al.*, 1999; Li *et al.*, 2003), the practical production is still problematic by the complexity of reactions and low yield of chemical synthesis. Therefore, it was considered that the production of dioscin via *in vitro*

culture is necessary.

In this study, we tried to produce dioscin from suspension culture of *S. china*. For *in vitro* mass production of dioscin, establishment of adventitious root culture is essential. But, to the best of our knowledge, no culture systems have been reported for *S. china*. Therefore, we have established an adventitious roots formation protocol from *S. china* and liquid culture of adventitious roots have been shown to have a potential for mass production of dioscin.

Materials and Methods

Plant material and primary culture

The plants of *Smilax china* L. were collected from the mountains of the Andong and Bongwha regions, Kyungpook, Korea. The young and soft leaves were immersed in aseptic water, and immersed in 70% ethanol for 1 min, sterilized in 3% H_2O_2 for 3 min, 2% NaOCl for 15 min and then rinsed five times with aseptic distilled water.

The sterilized leaf of *S. china* were inoculated into petri-dishes containing 20 mL of MS medium (Murashige and Skoog, 1962) containing $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $10 \text{ g} \cdot \text{L}^{-1}$ agar

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and different concentrations of kinetin and NAA. After 6 weeks cultivation at 27°C at dark condition, the ratios of callus formation and rooting were recorded.

Induction of adventitious roots by liquid culture

The root explants cultured on solid medium were used to induce adventitious roots using MS basal medium containing 30 g·L⁻¹ sucrose and different concentrations of kinetin and NAA; three concentrations of NAA (0, 1.0, 2.0 mg·L⁻¹) in combinations with 0, 0.1, 1.0 mg·L⁻¹ of the kinetin were tested. For selection of high dioscin production strain, the cultivation was started by inoculation of 100 mg of sliced root segments, which were induced from different plants, into 250 mL erlenmeyer flask containing 50 mL medium with continuous shaking of 100 rpm. After 6 weeks cultivation at 27°C, the length of adventitious root, the number of new adventitious root formation and the fresh weight of adventitious roots were recorded.

Analysis of dioscin content

Plant samples were dried at 60°C for at least 24 hours and two grams of dried samples were extracted with 100 mL of methanol by sonic vibrator at 50°C for 8 hours, and the extracts were injected into HPLC analysis system (SCL-10A system controller, LC-10 AD pump, SPD-10A UV-218 nm detector, Shimadzu, Japan and Nova-Pak C18 column, Waters, U.S.A.). The mobile phase for HPLC consisted of 60% acetonitrile (v/v) with a flow rate of 1 mL·min⁻¹. The retention times and detection limits for dioscin was 5.7 min, and 20 mg·mL⁻¹, respectively.

Results and Discussion

Callus and root induction

In order to induce roots from leaf explants, *in vitro* culture of *S. china* L. was conducted in MS medium supplemented with different concentrations of kinetin and NAA. As shown in table 1, the effects of NAA and kinetin concentration were significant on callus induction and rooting. An increase in the concentration of NAA from 1.0 to 2.0 mg·L⁻¹ promoted callus induction and rooting from leaf explants. Beside this the callus inductions were best at 0.1 mg·L⁻¹ kinetin + 2.0 mg·L⁻¹ NAA, and rooting was best at 1.0 mg·L⁻¹ kinetin + 2.0 mg·L⁻¹ NAA. As the 0.1 mg·L⁻¹ of kinetin added, the rooting was increased as compared to single application of NAA. The *in vitro* culture of stem explants of *S. china* L. was also conducted in the same concentration of kinetin and NAA, and the ratios of rooting and callus induction were also recorded. However, rooting efficacy was lower than that from leaf explants (results not shown).

The dioscin contents of callus root were measured. Dioscin was detected only in root, not in the leaf, stem or callus. The average dioscin content was 8.96±6.81 mg in gram of dried root which were induced from leaf explants. These results suggest that the dioscin production is only active in root.

Adventitious root culture

The sliced root segments which were cultured on MS solid medium were harvested and inoculated into MS liquid medium containing different concentrations of kinetin and NAA. As shown in table 2, the addition of kinetin was

Table 1. Effect of kinetin and NAA on rooting and callus formation from the leaf of *Smilax china* L.

Treatments (mg·L ⁻¹) Kinetin + NAA	Callus formation (%)	Root formation (No./petridish)
0 + 0	0.0 a	18.0 a
0 + 1.0	14.7 b	22.6 a
0 + 2.0	50.7 c	60.8 b
0.1 + 1.0	20.3 b	84.6 b
0.1 + 2.0	57.8 c	104.3 bc
1.0 + 1.0	7.8 b	101.6 bc
1.0 + 2.0	25.5 b	135.6 c

The 20 leaf segments with size of 5 x 5 mm were cultured in 9 cm petridish containing MS medium supplemented with 3% sucrose and incubated at 27°C for 6 weeks. Values are mean of ten replications and means followed by DMRT at the 5% level.

Table 2. Effect of kinetin and NAA on the adventitious root formation from the roots explants of *Smilax china* L.

Treatments (mg·L ⁻¹) Kinetin + NAA	New adventitious roots		
	Number (No. · cm ⁻¹ root)	Length (mm)	Fresh weight (mg/flask)
0 + 0	0.0 a	-	-
0 + 1.0	3.6 a	55.0 c	100.7 a
0 + 2.0	14.3 b	20.6 b	657.8 b
0 + 3.0	12.6 b	7.0 a	156.7 a
0.1 + 1.0	4.3 a	48.8 c	311.0 ab
0.1 + 2.0	22.6 c	22.0 b	1,118.5 c
0.1 + 3.0	20.0 c	7.0 a	675.7 b

The 100 mg of root segments were initially inoculated into 250 mL Erlenmeyer flask containing 50 ml MS liquid medium containing 3% sucrose. The flasks were maintained at gyratory shaker at 100 rpm, at 27°C for 6 weeks. Values are mean of ten replication and followed by DMRT at the 5% level.

significant for increasing number of new adventitious root, but adventitious root length was not affected by kinetin treatment. The effect of NAA concentration was significant for the formation of new adventitious root and increment of biomass. Optimal adventitious growth were achieved at the concentration of 0.1 mg·L⁻¹ kinetin + 2.0 mg·L⁻¹ NAA. However, length of adventitious root was decreased as the concentration of NAA increased (Table 2).

Therefore, the suspension cultures of adventitious root were conducted in MS liquid medium containing 30 g·L⁻¹ of sucrose supplemented with 0.1 mg·L⁻¹ kinetin + 2.0 mg·L⁻¹ NAA. Chemical structure of dioscin and adventitious roots harvested from 6 weeks suspension culture are shown in Fig. 1.

Selection of dioscin high production strain

To select an efficient strain for higher production of dioscin, the 200 mg of the twenty adventitious roots induced from different plants were inoculated into 250 mL flask containing 50 mL of liquid medium. After 6 weeks, the content of dioscin in adventitious root was analyzed. The contents of dioscin in twenty different strains were varied from 0.2% in strain No. 1, 2 to 2.6% in strain No. 10, without relevance to the color of culture broth or the biomass increases (Fig. 2). Although we don't have a reasonable explanation for severe variation of dioscin production in different adventitious roots, the production ability of dioscin from each root strain was maintained during several times of subculture.

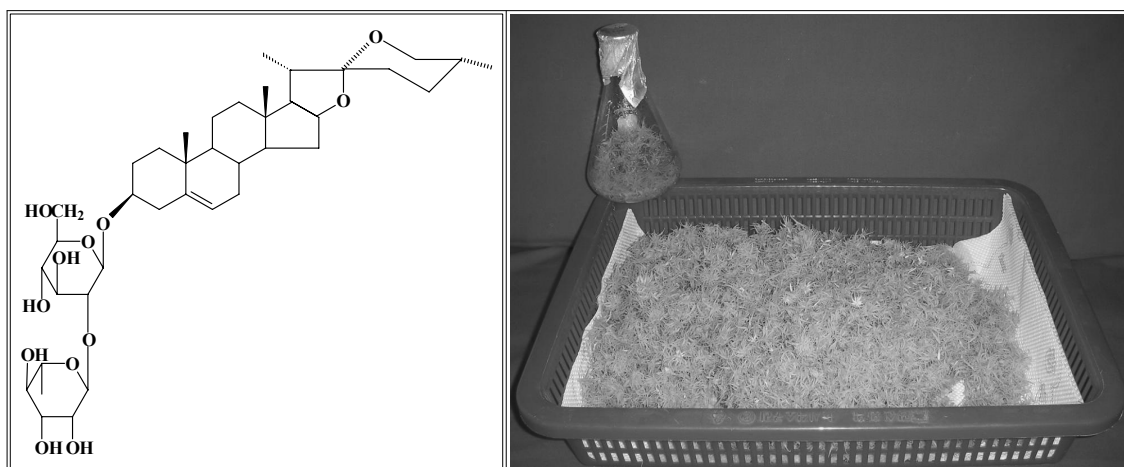


Fig. 1. Chemical structures of dioscin identified in *Smilax china* L.(left), and mass production of adventitious root from the roots explants of *S. china* L(right). by MS medium with 0.1 mg·L⁻¹ kinetin + 2.0 mg·L⁻¹ NAA.

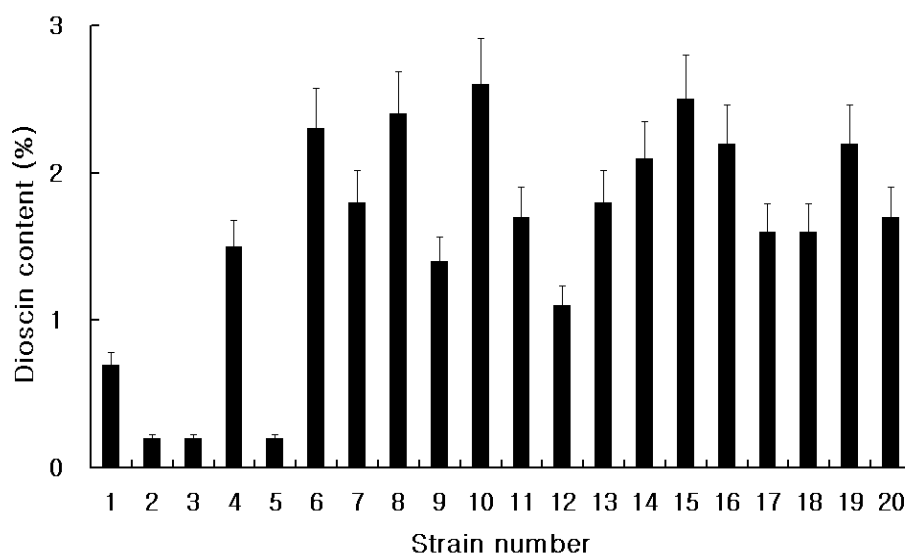


Fig. 2. The contents of dioscin in the twenty different cultures of adventitious roots formed from different strains of *Smilax china* L.

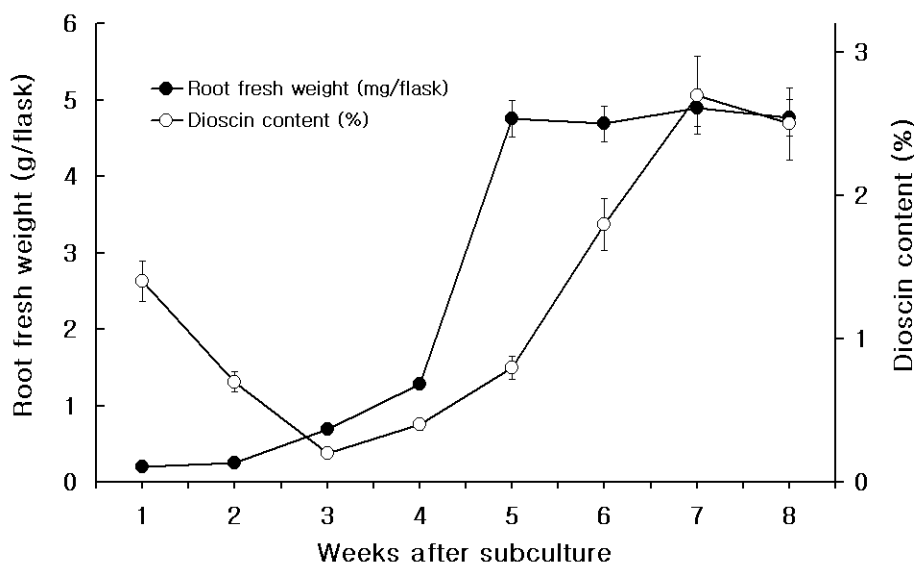


Fig. 3. The changes of contents of dioscin, and increase of biomass during 8 weeks cultivation of strain No. 10. The 200 mg of adventitious root segments were initially inoculated in 250 mL erlenmeyer flask containing 50 mL MS medium with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and $0.1 \text{ mg} \cdot \text{L}^{-1}$ kinetin + $2.0 \text{ mg} \cdot \text{L}^{-1}$ NAA.

The increases of biomass and the contents of dioscin of strain No. 10 were measured for 8 weeks (Fig. 3). The biomass was exponentially increased between 4 to 5 weeks, and maintained after 5 weeks; the inoculated 200 mg was increased to 4,895 mg at 8 weeks, an increase of about 24.5 folds. The contents of dioscin was less than 1% of dry root until 4 weeks. However, their contents were sharply increased from 5 weeks and reached 2.7 % at 7 weeks after culture. The rapid increases of dioscin during stationary

phase suggesting that the dioscin is secondary metabolites, and these contents may be controlled by environmental changes. Also, these results are coincided with the previous report that the synthesis of diosgenin is observed in stationary phase in *D. deltoids* (Tal and Goldberg, 1982) and *Dioscorea nipponica* (An *et al.*, 2005).

Considering a mass-production of dioscin from large scale culture such as continuous or bioreactor culture, the stain No. 10 is prominent. Further researches on the mass-production

technology such as, elucidation of production pathways for dioscin and the relevant genes, increased production by elicitation, and large-scale cultivations are necessary.

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