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Mass Production of Adventitious Roots of *Eleutherococcus sessiliflorus* through the Bioreactor Culture

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Abstract

This paper reported the establishment of mass production system of adventitious roots of *Eleutherococcus sessiliflorus* through the shake flask and bio-reactor culture. Induction of adventitious roots was started from the explants of germinated somatic embryos on half-strength Murashing and Skoog (MS) solid medium. The frequency of adventitious root formation was better in the explants comprising the basal hypocotyl parts than root explants alone. Among the different auxins tested (NAA, IBA and IAA), frequency of adventitious root induction was highest on medium with 0.5 mg/L NAA, and produced 16.3± 1.9 roots per explant. In shake-flask culture, deletion of NH_4NO_3 of MS medium was effective for induction of adventitious root compared with both full and half-strength MS media. Fresh weight increase of induced adventitious roots was performed well in medium with 0.5 mg/L IBA. When adventitious roots produced in shake-flask culture were transferred to 10-liter bioreactor, 5.5 times of fresh weight increase was gained after one month of culture. HPLC analysis revealed that the amount of eleutheroside E and E1 was higher in *in vitro* cultured adventitious roots than the 3 year-old field cultivated root barks of *Eleutherococcus sessiliflorus*. The content of eltutheroside B was much lower in adventitious roots than that of field cultivated one.

Key words: Eleutheroscoccus, eleutheroside, bio-reactor, adventitious root

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Introduction

Eleutherococcus (or *Acanthopanax*) species are important medicinal woody plants belonging to the Araliceae, and distributed mainly in northeastern Asia (Lee 1979). The cortical tissues of roots are used for medicinal purpose, primarily for their tonic and adeptoqenic action (Brekhman and Dardymov 1969). Propagation of the plants by seed is difficult because over 18 months of stratification are required for germination of zygotic embryos (Isoda and Shoji 1994). Rooting of stem cuttings and division of roots are the main ways of propagation but their efficiency is low (Ahn and Choi 1993). Many species of *Eleutherococcus* are endangered because of excessive random harvest.

Plant cell and tissue culture technique can be an effective method for production of plant materials using bioreactor. *In vitro* produced hairy and adventitious roots can display the similar biosynthetic capabilities to those of natural roots (Yoshikawa 1987). In *Panax ginseng*, there are numerous publications on mass production of adventitious (Paek et al. 2000) and hairy roots (Choi et al. 2000). In *Eleuthereococcus*, somatic embryogenesis and plant regeneration have been reported (Choi et al. 2002). However, there is no report on the *in vitro* production of adventitious roots and hairy-roots in *Eleucherococcus species*.

This work was carried out to develop a system of the mass production of adventitious roots of *E. sessiliflorus* through *in vitro* culture system.

Materials and Method

Somatic embryo-derived plantlet production

Embryogenic cells of *Eleutherococcus sessiliflorus* Rupr. et Maxim induced by Choi et al. (2002) were maintained on MS

solid medium with 4.5 μM 2.4-D and 3% sucrose by 2-week subculture intervals. To induce somatic embryos, embryogenic callus was transferred to MS solid medium lacking 2,4-D in 10 ×2 cm petri dishes containing 30 mL MS solid medium. After induction of cotyledonary embryos, these embryos were transferred on to MS solid medium (0.3% gelrite) with 3% sucrose and 20 μM GA_3 to induce germination. After 3 weeks of culture, germinated embryos (3-4 cm in length) were ready for the source of the explants. The pH of medium was adjusted to 5.8 before autoclaving (15 min, 121°C, 1.2 kg/cm^2). They were cultured in complete dark or light condition with a 16-h photoperiod of 24 μmol $m^{-2}s^{-1}$(cool white fluorescent tubes). Culture room was maintained at 20±2°C.

Adventitious roots induction on solid medium

Four types of explants (roots, root with basal portion of hypocotyls, hypocotyls and cotyledon) excised from germinating somatic embryos were cultured on 1/2 MS solid (0.3% gelrite) medium, with 3% sucrose and 0.5 mg/L NAA in 10×2-cm plastic petri dishes containing 30 mL of medium. Twenty explants were used in each experiment, and this was repeated three times. After 4 weeks of culture, frequency of adventitious roots formation was examined.

To evaluate the effect of auxin, roots with hypocotyls were cultured on 1/2 MS solid medium with 10 g/L sucrose and three auxins (NAA, IBA and IAA) at different concentrations (0.1, 0.5 and 1.0 mg/L). Five explants were cultured in each plastic petri dish. About 20 explants were cultured in each experiment. This treatment was repeated three times. After four weeks of culture, the number and length of adventitious roots were assessed.

Effect of nitrogen source in liquid culture

Roots with hypocotyls were cultured in variously modified MS liquid medium with 0.5 mg/L NAA or 0.5 mg/L IBA (MS medium lacking NH_4NO_3, MS medium with half strength NH_4NO_3 and KNO_3, 1/2-strength MS medium and full-strength MS medium). Twenty explants were cultured in each 100 mL Erlenmeyer flask and the experiment was repeated three times. After 4 week of culture, frequency of adventitious root formation was observed.

Fresh weight increase of adventitious root in liquid culture

Adventitious roots formed in MS liquid medium lacking NH_4NO_3 with 0.5 mg/L IBA were transferred to 100 mL Erlenmeyer flask containing 25 mL of the same medium with 1% sucrose and various levels of NAA, IBA or IAA (0.1 mg/L, 0.5 mg/L, 1 mg/L). Initial inoculum of adventitious roots was adjusted to 0.5 g. After one month of culture, fresh weight of adventitious roots was measured. The liquid culture was performed at 20±2°C in the dark at 110 rpm on a rotary shaker.

Culture in bioreactor

Adventitious roots produced in 250 mL Erlenmeyer flask culture with 0.5 mg/L IBA were cut to 2 cm in size, and then inoculated in 10-liter air bubble balloon-type bioreactor containing 3-liter 1/2 MS medium without NH_4NO_3 and with 0.5 mg/L IBA or lacking auxin. Initial root inoculum was adjusted to 20 g for the bioreactor culture. After one month of culture, fresh weight of adventitious roots was measured.

HPLC analysis

Dried powders of *in vitro* cultured adventitious and field cultivated roots (1 g) were extracted with 70% ethanol (50 mL) in 80°C, refluxed for 1h, twice, and filtered through filter paper (Advantec, Toyo, Japan). The EtOH extract was evaporated to dryness and dissolved in 50 mL of HPLC grade water. The water fraction was washed two times with the same volume of ether. The water fraction was evaporated to dryness and dissolved in 5 mL of 70% ethanol and filtered through 0.45 μm PTEF filter (Gelman, USA). The eleutherosides fraction was analyzed by using a HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millenium 2010 chromatography manager) on a NovaPak C18 column (3.9×150 mm, 4 μm), with water and acetonitrile. The rate of water and acetonitrile for initial, 10, 30, 40, 45, 46 and 50 min, were 95:5, 90:10, 60:40, 50:50, 45:55, 95:5 and 95:5, respectively. Flow rate of the mobile phase was 0.8 mL/min and monitoring of eleutherosdies was 220 nm. The authentic eleutherosdie B, E and E1 were purchased from Chromadex Inc., USA. The total eleutherosides content was calculated as the sum of eleutherosides fractions.

Results and Discussion

Adventitious root formation from different explants

Four kinds of explants (roots, root with hypocotyls, hypocotyls and cotylendons) excised from germinating somatic embryos of *E. sessiliflorus* were cultured on 1/2-strength MS solid medium with 0.5 mg/L NAA for four weeks. Roots comprising basal parts of hypocotyls were the most effective explants for adventitious root induction than others (Table 1). New roots were induced laterally on the surfaces of cultured roots. About 17

adventitious roots were formed per explants (Table 1), whereas, no lateral root was formed on medium without exogenously supplied auxin (Figure 1). Cotyledon explants did not produce any adventitious roots even on medium with auxin. The highest frequency of adventitious root production from root explants comprising basal parts of hypocotyls revealed that the hypocotyl parts plays a role for promotion of adventitious root formation although the detailed mechanism is not yet known.

Complete dark culture was more effective for the production of adventitious roots than light illumination culture (Table 1). This result was agreed to the ginseng root culture that induced more hairy root and adventitious roots in dark condition than in light condition (Yang et al. 1996).

Effect of exogenous auxins on adventitious root production

Root explants comprising hypocotyls were cultured on 1/2-strength MS solid medium supplemented with various auxins (NAA, IBA and IAA) at different concentration (0.1, 0.5 and 1 mg/L). NAA was the best auxin for the adventitious root induction compared to IBA and IAA (Table 2). However, adventitious root formation was suppressed markedly on medium with more than 1.0 mg/L NAA, and instead the explants were callused after 3 weeks of culture. On medium with 0.5 mg/L IBA or 0.5 mg/L IAA, elongation of adventitious roots was rapid although the frequency of adventitious root formation was low (Table 2). These results suggest that the effect of auxin on adventitious root induction and elongation was depended on the plant types. In general, high level of auxin promoted the production of adventitious roots, although the auxin inhibited the elongation of root (Blakesley et al. 1991). Contrary to our result, in *Malus* (Geert-Jan et al. 1997), IAA was effective for the production of adventitious roots and NAA strongly inhibited the growth of adventitious roots.

Table 1. Formation of adventitious roots from different explants of germinating somatic embryos of *E. sessiliflorus* in 1/2 MS solid medium supplemented with NAA 0.5 mg/L in dark or light after one month of culture.

Explant	Dark		Light	
	No. of adventitious roots	Length of adventitious roots (mm)	No. of adventitious roots	Length of adventitious roots (mm)
Root	12.7±2[a]	2.8±0.5	7.5±1.7	1.6±0.5
Root+hypocotyl	16.3±1.9	4.5±1.2	11.1±1.8	2.3±0.5
Hypocotyl	2.5±0.5	4.6±0.4	2.7±0.4	3.5±0.4
Cotyledon	0	0	0	0

[a]Data represent the mean values±SE from three independent experiments.

Table 2. Effect of auxin on adventitious root formation from explants of roots with hypocotyls of *E. sessiliflorus* in 1/2 MS solid medium after one month of culture in dark or light.

Auxin (mg/L)	Dark		Light	
	No. of adventitious roots	Length of adventitious roots (mm)	No. of adventitious roots	Length of adventitious roots (mm)
Control	0	0	0	0
NAA 0.1	8.4±2.5[a]	6.2±0.9	7.9±1.7	4.5±0.6
0.5	16.3±1.9	4.5±1.2	11.0±1.8	2.3±0.5
1.0	2.4±0.6	2.5±0.4	2.2±0.8	1.9±0.4
IBA 0.1	5.0±1.0	6.8±0.9	3.5±0.9	5.0±0.6
0.5	10.2±2.3	7.7±1.1	8.5±2.4	6.5±0.8
1.0	1.7±0.2	3.5±0.3	1.4±0.5	2.5±0.3

[a]Data represent the mean values±SE from three independent experiments.

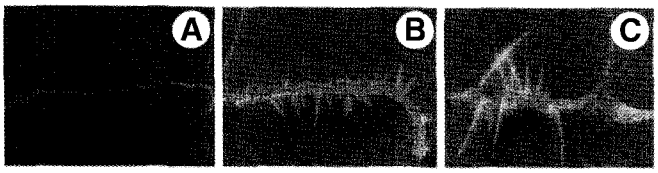

Figure 1. Adventitious root formation on solid medium with different auxin. (A) 1/2 MS medium without auxin. (B) 1/2 MS medium with NAA 0.5 mg/L. (C) 1/2 MS medium with IBA 0.5 mg/L.

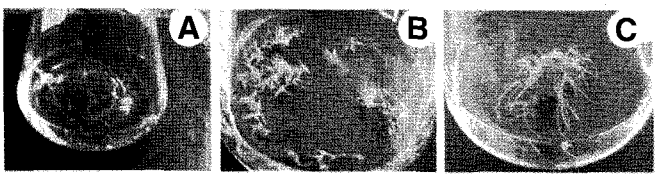

Figure 2. Formation of adventitious roots from root explants in 100 mL Erlenmeyer flasks containing modified MS liquid medium without NH_4NO_3. (A) Medium lacking auxin. (B) Medium with NAA 0.5 mg/L. (C) Medium with IBA 0.5 mg/L.

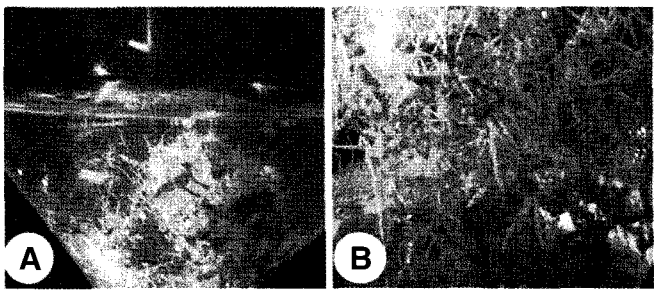

Figure 4. Bioreactor culture of adventitious roots. (A) Adventitious roots cultured in 10-L bioreactor in modified MS liquid medium without NH_4NO_3 and with 0.5 mg/L IBA. (B) Closed view of adventitious roots.

Effect of nitrogen sources on adventitious root production

Root explants with basal parts of hypocotyls were cultured in modified MS liquid media; 1/2-strength MS medium, MS medium lacking NH_4NO_3, MS medium with half strength NH_4NO_3 and KNO_3. In MS medium lacking NH_4NO_3, the frequency of adventitious roots production was 2-3 times higher than the other media, and about 22-23 adventitious roots were formed per explants (Table 3). In addition, total fresh weight of induced adventitious roots was highest in MS medium lacking NH_4NO_3 than the other media (Table 3). This result indicates that adventitious root formation was stimulated when NO_3^- was used to the sole nitrogen source and high concentration of ammonium salts might suppress the formation of adventitious roots. Similar result has been reported that a low level of ammonium stimulated the formation of adventitious roots in cotyledon culture of *Panax ginseng* although a high level of ammonium fostered the formation of both somatic embryos and embryogenic calli (Choi et al. 1997). In adventitious root culture of *Panax ginseng*, high levels of NH_4^+ ion inhibited the growth of adventitious roots (Yu et al. 2000).

Effect of nitrogen sources

To obtain the continuous production of induced adventitious roots in shake flask culture, adventitious roots induced in MS liquid medium lacking NH_4NO_3 in the presence of 0.5 mg/L IBA were transferred to the same liquid medium with NAA, IBA or IAA at different concentration (0.1, 0.5 and 1 mg/L). Increase of fresh weight of adventitious roots was best in medium with IBA compared to NAA or lacking auxin (Figure 3). In liquid medium with 0.5 mg/L IBA, fresh weight was 3.3±0.4 g, and this showed a 6-fold increase of fresh weight after one month of culture. In medium with NAA, adventitious roots were turned brown without both elongation and new root formation. This result indicates that the requirement of effect auxin concentration is different between the solid and liquid culture.

Table 3. Induction of adventitious roots of *E. sessiliflorus* in modified MS liquid media with NAA 0.5 mg/L and IBA 0.5 mg/L after 4 weeks of culture.

Medium	NAA 0.5 mg/L		IBA 0.5 mg/L	
	Number	FW (mg)	Number	FW (mg)
MS	3±0.3[a]	326±17	2±0.1	367±47
MS without NH_4NO_3	21±1.5	1255±137	23±2.3	1539±217
MS with 1/2 NH_4NO_3 + KNO_3	10±1.6	533±37	11±1.2	652±97
1/3 MS	17.2±1.5	725±123	15±1.7	876±114

[a]Data represent the mean values±SE from three independent experiments.

Mass production of roots in bioreactor

Twenty grams of adventitious roots produced in shake flask culture were transferred to 10-liter balloon-type air bubble bioreactor containing 3-liter 1/2 MS medium lacking NH_4NO_3. Fresh weight was increase at 5.5 times (110 g) after one month of culture (Figure 4. A-B), while 3.7 times (74 g) increase of fresh weight was gained in hormone-free 1/2 MS medium lacking NH_4NO_3.

Eleutheroside analysis

HPLC analysis revealed that there were some differences between field-cultivated roots and *in vitro* produced adventitious roots. *In vitro* produced adventitous roots contained higher amount of eleutheroside E and E1 compared to three years-old field cultivated roots, whereas the content of eleutheroside B was very low or not detectable in *in vitro* produced adventitious (Table 4). All the eleutheroside B, E and E1 were phenylpropane derivatives, most of which were glycosylated (Sonnenborn and Hänsel 1993). The low content of eleutheroside B might be caused from the volatile character in air bubble condition because this compound is derived from phenylpropane. However, Kang et al. (2001) reported that some of *Eleutherococcus* species did not show any peak corresponding to eleutheroside B analyzed by the reversed-phase HPLC. The low content of eleutheroside B in cultured roots remains to be cleared.

Acknowledgments

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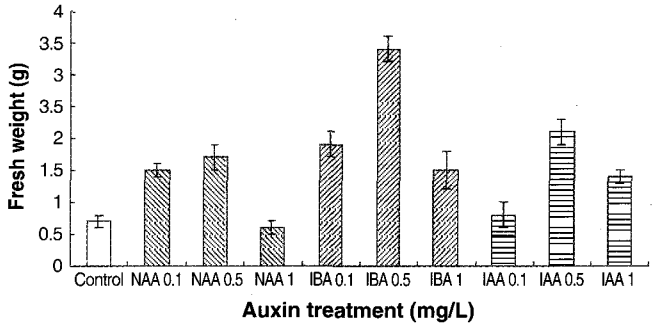

Figure 3. Growth of subcultured adventitious roots of *E. sessiliflorus* in medium with different auxin after one month of culture. Adventitious roots (0.5 g as initial inoculum) were cultured 100 mL Erlenmeyer flask containing MS liquid medium without NH_4NO_3. Data were colleted after one month of culture and represent the mean±the standard deviation.

Table 4. Eleutheroside content of adventitious roots of *E.sessiliflorus*.

Culture type	Explant	Eleutheroside		
		B	E	E1
In vitro liquid culture	Adventitious root	-	658.0±34.5	384.1±26.4
Wild cultivated	Root bark	229.5±17.2	344.2±27.6	164.2±8.9

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