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Micropropagation of Medicinal Woody *Eleutherococcus pedunculus* via Somatic Embryogenesis

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ABSTRACT : Zygotic embryos just after harvest of seeds were immature globular to heart stage. Maturation of zygotic embryos rapidly proceed when zygotic embryos together with small excised parts of endosperm were cultured on 1/3-strength MS solid medium with 2% sucrose, and the zygotic embryos were germinated within two months. Embryogenic callus was formed from the excised segments of germinating zygotic embryos of *Eleutherococcus pedunclus* on Murashige and Skoog (MS) medium with 4.5 μM 2,4-D. The embryogenic callus formation occurred at a low frequency (less than 7%) from hypocotyl segments. The embryogenic calli were maintained on the same medium as primary medium. High frequency somatic embryogenesis was obtained after the cells were transferred to medium lacking 2,4-D. Cotyledonary embryos were germinated and converted into plantlets on medium with 20 μM GA_3. Embryogenic callus and somatic embryos were produced spontaneously on the surfaces of roots and/or hypocotyls of plantlets. The frequency of embryogenic callus formation was 85% in roots and 34% in hypocotyls. Therefore maintain of cell lines performed very easily. Plantlets with developed epicotyls at more than 3 cm acclimatized at high frequency (89%). While plantlets with small epicotyls (less than 1 cm) were acclimatized at low rate (32%). The soil survived plantlets produced new sprouts after over wintering in the field.

Keywords : *Plant regeneration*, *Acanthopanax*, cell culture

Abbreviations : MS - Murashige and Skoog; GA_3 - Gibberellic acid; 2,4-D - 2,4-dichlorophenoxy acetic acid

INTRODUCTION

Eleutherococcus (or *Acanthopanax*) *species* are important medicinal woody plants, belonging to the Araliceae (Lee, 1979). *E. pedunculus* is distributed only in Korea and endangered because of excessive random harvest (Yook, 1995). The cortical tissues of its roots and stems are used for medicinal purposes, primarily for their tonic and adaptogenic action (Brekhman and Dardymov, 1969). Plants of *Eleutherococcus* have to be sacrificed at the time of root harvest. Propagation of the plants by seeds is difficult because over 18 months is required for both maturation and germination of zygotic embryos (Isoda and Shoji, 1994). Rooting of stem cutting and division of roots are the main ways of propagation but their efficiency is low (Ahn and Choi, 1993). Thus, plant tissue culture should be applied to as a propagation method for *Eleutherococcus*. Micro propagation of this plant has not been reported yet. Plant regeneration through somatic embryogenesis has been reported for *E. senticosus* (Choi *et al*., 1999a,b; Gui *et al*., 1991) and *E. sessiliflorus* (Choi *et al*., 2002).

In this paper, we report for the first time the micro propagation of endangered *E. pedunculus* via high frequency somatic embryogenesis and successful transfer of plantlets to soil.

MATERIALS AND METHODS

Embryogenic callus and somatic embryo induction

After dehiscence, the dehusked seeds of *Eleutherococcus pedunculus* were immersed in 70% ethanol for 1 min,

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sterilized in 1% sodium hypochlorite for 1hr and rinsed 3 times with sterile distilled water. To examine the maturation of zygotic embryos, dehusked seeds, small excised part of seeds including embedded zygotic embryos, and isolated zygotic embryos were on half-strength MS (Murashige and Skoog, 1962) medium containing 1% sucrose and 0.25% gelrite. Maturation of zygotic embryos was noted during 2 months of culture. Germinating zygotic embryos (10 mm in length) after 2 weeks of culture were transversely cut into 2 mm segments (cotyledons, hypocotyls and roots) that were then placed on the surface of the medium. The medium was MS medium containing 4.5 μM 2,4-D, 3% sucrose and 0.25% gelrite in 10×2 cm plastic Petri dishes containing 30 ml of medium. The medium was adjusted to pH 5.8 before autoclaving at 120℃ for 15 min. The culture room was maintained at 24℃ with a 16-h photoperiod of 24 μmol $m^{-2}sec^{-1}$ (cool white fluorescent tubes). Fifteen explants were cultured in each Petri dish with three replicates and the experiment was repeated three times. After 2 months of culture, the frequency of somatic embryo formation and embryogenic callus formation was assessed.

Embryogenic callus was maintained on MS solid (0.25% gelrite) medium with 4.5 μM 2,4-D and 3% sucrose and sub-cultured at 4-week intervals. To maintain via cell suspension culture, embryogenic callus was transferred to MS liquid medium with 4.5 μM 2,4-D and 3% sucrose and sub-cultured at 2-week intervals. A 200 mg sample of cells was transferred to each of several 250 ml Erlenmeyer flasks containing 50 ml liquid medium. The cultures were agitated at 100 rpm on a gyratory shaker under 12 μmol $m^{-2}sec^{-1}$ cool white fluorescent light with a 16-h photoperiod.

Germination and plant conversion

When somatic embryos developed to the globular stage after two weeks of culture, about 300 embryos were plated on each Petri dish containing solid hormone-free MS medium (0.25% gelrite). To induce germination and plant conversion of embryos, cotyledonary embryos were transferred to MS solid medium (0.25% gelrite) with 3% sucrose and 20 μM GA_3. After one month, plantlets were transferred to 1/3 MS solid medium with 1% sucrose in 6×7 cm glass bottles to support further growth.

Plantlets with different size of epicotyls (1, 2, and 3 cm in length) were transferred to square plastic pots containing perlite and peat moss (1 : 1, V/V). The pots were covered with polyvinyl for one month. During the acclimatization, plantlets were kept at 24℃ for one month. The pots minus their cover were transferred to the glass house for two months. Survival of plants was recorded after two months of cultivation. Twenty plants were planted in soil in each experiment, and each experiment was repeated three times. Surviving plantlets were transferred to the field, and exposed to winter. In the following spring, sprouting from the stems was examined.

RESULTS AND DISCUSSION

Rapid maturation of zygotic embryos

Zygotic embryos just after harvest were highly immature globular to heart-shaped stage (less then 300 μm in size). Naturally at least more then 18 months of stratification was required (Isoda and Shoji, 1994). When small excised part of seeds including embedded zygotic embryos were on half-strength MS medium containing 1% sucrose and 0.25% gelrite, maturation of zygotic embryos to the cotyledonary stage were performed after one month of culture (data not presented). Isolated zygotic embryos did not proceed to mature probably caused from suitable culture condition. While, in the culture of dehusked intact seeds, maturation of zygotic embryos was very slow and similar to the natural stratification treatment. The above results suggest that there are unknown substances in the seeds suppressing the maturation of zygotic embryos.

Embryogenic callus induction

When excised segments of germinating zygotic embryos of *E. pedunculus* were cultured on MS agar medium with 4.5 μM 2,4-D, somatic embryos developed directly on the surface of explants and/or embryogenic callus were formed on the excised margin of explants after one months of culture. After 2 months, the frequency of direct embryo

formation was the highest in the hypocotyl segments (72%) and lowest in the root segments (8%) (Table 1). After two months of culture, the hypocotyl explants produced a small amount of friable and yellow-brown embryogenic callus at the frequency of 7% (Table 1). *E. senticosus*, seedling explants never produced embryogenic callus in the presence of 2,4-D (Gui *et al*., 1991 and Choi *et al*., 1999a), thus embryogenic callus was induced only by consecutive subculture of somatic embryos (Choi *et al*., 1999b). *E. pedunculus* germinating zygotic embryos can produce embryogenic callus by consecutive subculture although the frequency was low.

Somatic embryo formation from embryogenic callus

Embryogenic calli were transferred to MS solid medium with 4.5 μM 2,4-D (Fig. 1A) and were maintained through 2 week subculture intervals on the same medium. When embryogenic calli were transferred to MS medium lacking 2,4-D, a large number of globular embryos formed after 2 weeks of culture (fig. 1B). The average number of embryos per 200 mg of cell inoculum was 1750. The density of embryos was too many for proper development in the Petri dish and therefore, globular embryos in a Petri dish were divided to three Petri dishes containing hormone- free solid MS medium. Development of embryos proceeded to the cotyledonary stage within one and two months (Fig. 1C-D).

Plantlet conversion

To induce germination and plant conversion of embryos, cotyledonary embryos were transferred to MS solid medium (0.25% gelrite) with 3% sucrose and 20 μM GA_3 (Fig. 1E). In *E. pedunculus*, 100% of cotyledonary embryos converted into plantlets with well-developed shoot and roots (Fig. 1F). Production and plant conversion of embryos of *E. pedunculus* were as efficient as the embryogenic culture of carrot, known as a model plant.

After one month, plantlets were transferred to 1/3 MS solid medium with 1% sucrose in 10×10 cm plastic culture bottles to support further growth (Fig. 1D).

Table 1. Frequency of direct somatic embryo and embryogenic callus formation on different portions of seedlings of *E. pedunculus* on MS medium with 4.5 μM 2,4-D after 2 months of culture

Frequency of direct Explants	Embryogenic callus	
	embryo formation (%)	formation (%)
Cotyledon	56±12[a]	0
Hypocotyl	72±19	7.8±0.9
Root	8±1.2	3.5±0.5

[a]Data represent the mean values ± standard error of three independent experiments

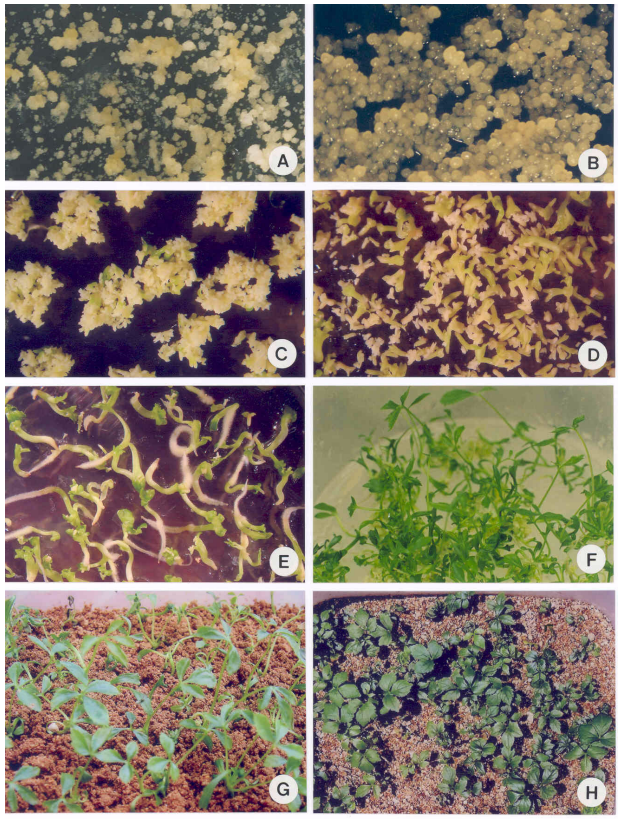

Fig. 1. Plant regeneration via somatic embryogenesis from cultures of *E. pedunculus*. A: Friable embryogenic callus maintained on MS medium with 4.5 μM 2,4-D. B: Globular embryos formed on MS medium lacking 2,4-D after two weeks. C: Numerous cotyledonary embryos formed from embryogenic callus on MS medium lacking 2,4-D after 5 weeks of culture. D: Cotyledonary embryos after plating on MS solid medium. E: Germinating somatic embryos on MS medium with 20 μM GA_3. F: Plantlets of *E. pedunculus* after transfer to 1/3 strength MS medium in plastic culture Jar. G: Plantlets acclimatized on soil of plastic container. H: New sprouting of soil transferred plantlets at next spring.

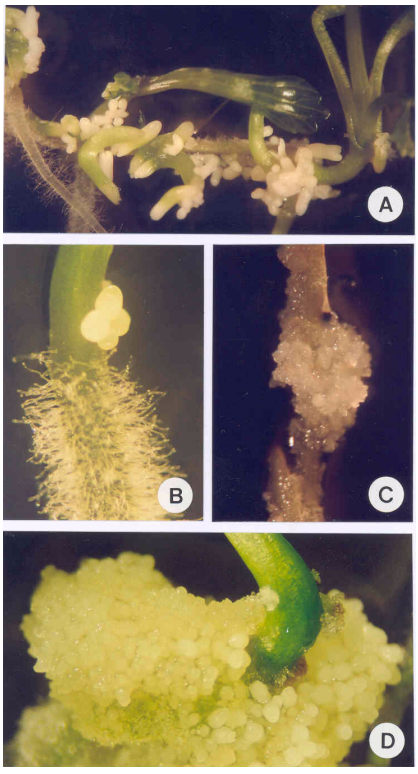

Fig. 2. Secondary embryo and/or embryogenic callus formation from somatic embryo-derived plantlets. A: numerous somatic embryos formed directly on the surfaces of hypocotyls and roots. B: Somatic embryos on hypocotyls of plantlets. C: Embryogenic callus formation on the surface of a root. D: Numerous somatic embryos developed from embryogenic callus.

Spontaneous induction from regenerated plantlets

Plantlets produced embryogenic callus and somatic embryos on the surfaces of roots and/or hypocotyls (Fig. 2). Cotyledon and hypocotyls produced very often the somatic embryos directly on the surfaces (Fig. 1A-B). Embryogenic callus frequently formed on the surfaces of roots of plantlets (Fig. 2C). The callus produced the numerous somatic embryos after culture times was proceed until two months (Fig. 2D). The frequency of somatic embryos and/or embryogenic callus formation was 85% in roots, 34% in hypocotyls, 12% in cotyledons, respectively. Therefore maintain of cell lines performed very easily.

Soil transfer

Developmentally different three stages of plantlets with different size of epicotyls (1, 2, and 3 cm) by the growth of plantlets were transferred to square plastic pots containing perlite and peat moss (1:1, V/V). The pots were covered with polyvinyl for one month. Frequency of soil survival of plants was higher (89%) in old plantlets with epicotyls at about 3 cm in height than the ones with epicotyls at 1 cm in length (Table 2, Fig. 1G). Surviving plantlets were transferred to the field, and exposed to winter. In the following spring, sprouting from the stems was performed (Fig. 1H).

Table 2. Effect of plantlet age on the frequency of soil survival of *E. pedunculus* after 2 months of culture

Size of Epicotyls (cm)	Height of plantlets (cm)	Survival of plantlets (%)
1	6	32±2.3[a]
2	8	53±6.7
3	10	89±11.2

[a]Data represent the mean values ± standard error of three independent experiments

In conclusion, commercial production of plantlets of *E. pedunculus* through somatic embryognesis appears to be possible.

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