

## High Frequency of Plant Regeneration through Cyclic Secondary Somatic Embryogenesis in *Panax ginseng*

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Somatic embryogenesis is one of good examples of the basic research for plant embryo development as well as an important technique for plant biotechnology such as medicinally important plants. Single embryos develop into normal plantlets with shoots and roots. Therefore, direct single embryogenesis derived from single cells is highly important for normal plant regeneration. Here we demonstrate that the cyclic secondary somatic embryogenesis in *Panax ginseng* Meyer is a permanent source of embryogenic material that can be used for genetic manipulations. Secondary somatic embryos were originated directly from the primary somatic embryos on hormone-free Murashige and Skoog medium, and proliferated further in a cyclic manner. EM medium (one third of modified MS medium [MS medium containing half amount of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub>] with 2% to 3% sucrose) favored further development of proliferated secondary somatic embryos into plantlets with root system. The plantlets developed into plants with well-developed taproots in half-strength Schenk and Hildebrandt basal medium supplemented with 0.5% activated charcoal.

**Keywords:** *Panax ginseng* Meyer, Regeneration, Somatic embryo, Cyclic secondary somatic embryogenesis

### INTRODUCTION

*Panax ginseng* Meyer is one of the medicinally important slow-growing perennial herb from *Araliaceae* family. It has been cultivated for its highly valued medicinal efficacy for more than thousand years in East Asia countries like China, Korea, and Japan [1]. Also, it is reported that saponin is one of the most important compounds in *Panax ginseng* [2]. During the stratification and germination, infection by pathogens or abiotic stresses can cause seed inviable [3]. In addition, absence of horticulturally improved varieties which are morphologically and genetically homogeneous strongly requires a clonal propagation method. It could contribute to its genetic improvement by reducing the generation cycle and the confounding genotype effect in field evaluations through

the use of clonal material.

Somatic embryogenesis has a great potential to aid crop improvement through large-scale clonal propagation, genetic transformation and synthetic seed production [4]. Somatic embryogenesis through secondary embryo possesses a multiplicative potential for clonal mass propagation [5]. During this secondary somatic embryogenesis, new somatic embryos are initiated from originally formed somatic embryos or primary somatic embryos. In addition, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis [6]. It is also reported that the efficiency of explants in secondary embryogenesis is higher than in primary embryogenesis [6]. High-frequency of

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plant regeneration systems through cyclic secondary embryogenesis has been reported in several plant species including black pepper [7], brassica [8], *Medicago truncatula* [9], Siberian ginseng (*Acanthopanax koreanum*) [10], cassava [11], and white clover [9], but there is no such report in *P. ginseng*. Direct somatic embryogenesis in *P. ginseng* has been reported from cotyledon explants. Cotyledon explants can produce somatic embryos directly on growth regulator-free medium developing as either multiple or single-state forms [10,12-16]. In ginseng, only single somatic embryos can regenerate into plants with well-developed roots and shoots, whereas the multiple and fused somatic embryos gave multiple shoots [13]. Improved regeneration through somatic embryogenesis is highly desirable due to the potential of embryogenic cultures to undergo repetitive somatic embryogenesis [17].

Inducing secondary embryo from primary embryo of ginseng can be useful method for a clonal propagation. It is limited to obtain zygotic embryo because one matured 4-year-old ginseng produce only 40 to 60 seeds, and it is also laborious getting embryo by removing seed coat one by one. Especially, regenerating plant by genetic transformation of ginseng is difficult, because single embryogenesis cannot be occurred easily on an antibiotic-containing hormone-free medium. In this present study, we report the establishment of a high-frequency plant regeneration system of *P. ginseng* through scale-up utilizing liquid culture and cyclic secondary embryogenesis, such as induction of secondary somatic embryogenesis, as well as proliferation, maturation, and germination.

## MATERIALS AND METHODS

### Plant material and explant preparation

*P. ginseng* cv. 'Yunpoong' seeds, provided by Ginseng Genetic Resource Bank, were used for somatic embryogenesis. Intact zygotic embryos (ZEs) were placed on Murashige and Skoog (MS) basal medium containing 3% sucrose and 0.7% agar to elongate, as described previously [16]. The growth culture room was maintained at 24±2°C with a 16-h (light)/8-h (dark) photoperiod with light being supplied by white fluorescent tubes at an intensity of 24 μmol/m<sup>2</sup>s<sup>-1</sup>.

### Induction of primary somatic embryos

To obtain primary somatic embryos (PEs), cotyledons excised aseptically from 4 d pre-cultured ZEs were placed abaxial side down on MS basal medium with or without 2,4-D (1 mg/L) and kinetin (0.1 mg/L). Cotyledons were cultured in 60×15 mm Petri dishes containing

15 mL of medium and transferred to fresh medium every month. Each treatment was replicated three times, and each replicate consisted of fifteen explants. The number of explants producing PEs was recorded at the end of 10th week of culture. Embryogenic clumps were maintained for 1 yr by subcultures at one month intervals to fresh EM medium (one third of modified MS medium [MS medium containing half amount of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub>] with 2% to 3% sucrose) in 40 mL of medium of 100 mL Erlenmeyer flask, and incubated in gyratory shakers. Aliquots of 4.5 g of somatic embryogenic callus were cultivated in each culture. For subsequent proliferation, fresh 7.3 g of embryogenic callus was subcultured in a 2 liter bioreactor containing 1.5 liter medium. Total fresh weight of embryo clump was measured after 30 d of culture.

### Induction of secondary somatic embryogenesis

To obtain a new cycle of secondary somatic embryogenesis, both pre-cotyledonary clumps and cotyledonary PEs were carefully detached and inoculated onto the EM (2% sucrose) or MS basal medium (3% sucrose). The medium was adjusted to pH 5.6 to 5.8 before autoclaving at 120°C for 15 min. Number of secondary somatic embryos (SEs) produced from PEs, and the percentage of produced SEs were recorded under a microscope (ICS-305B; Alphasytec, Seoul, Korea) per week after subculture.

### Germination and plant regeneration from secondary somatic embryos

SEs formed on MS basal medium were placed onto the EM medium. SEs were allowed to develop to heart stage, and then isolated from the mass culture for plant regeneration. To develop root of embryo-derived plantlets, germinated plantlets were transferred into development medium, half-strength Schenk and Hildebrandt (SH) basal medium including 0.5% activated charcoal. Rooted plants were potted in autoclaved mixture of peatmoss, vermiculate and perlite (1:3:1 v/v) in 5 plastic pots and covered with a transparent plastic top.

## RESULTS

### Primary somatic embryogenesis

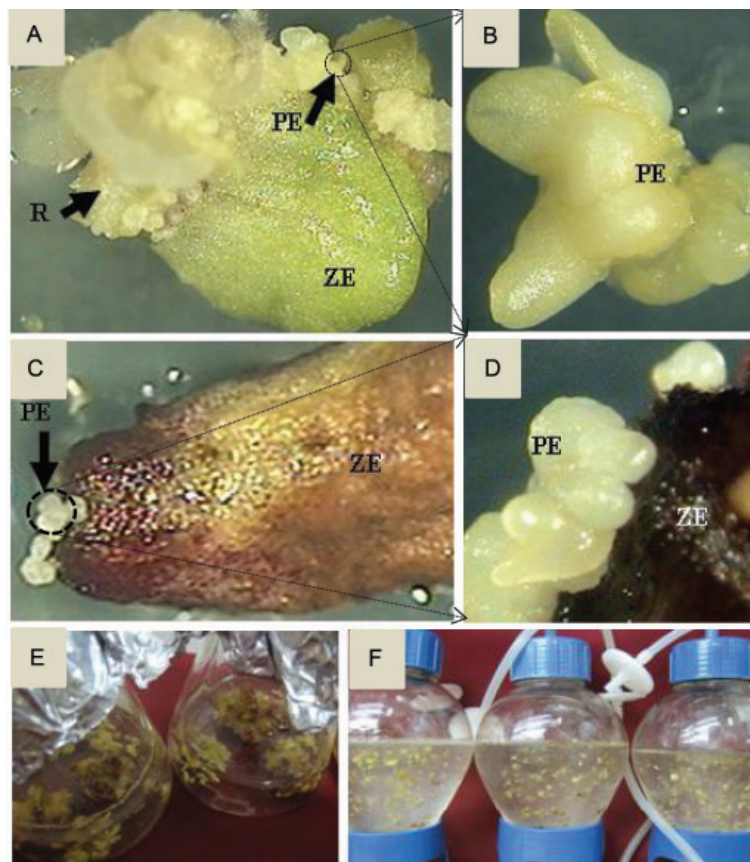
Total 32.5% of somatic embryos were produced from 60-day-cultured cotyledon explants, which cultured on MS medium without plant growth regulators (Table 1). Indirect somatic embryos were also formed on medium containing 2,4-D (1 mg/L) and kinetin (0.1 mg/L), and

**Table 1.** Ratio of generated somatic embryos from 60-day-cultured cotyledon explants, which were derived from Yunpoong zygotic embryo

Culture media in MS	Type and ratio (%) <sup>1)</sup>		Embryo type	Green color
Hormone-free	Embryo	32.5	Mostly single embryo	20%
	Root	2.5		
2,4-D (1 mg/L)+kinetin (0.1 mg/L)	Embryo	45.0	Mostly poly embryo	100%
	Root	62.5		
	Shoot	2.5		
	Callus	95.0		

MS, Murashige and Skoog.

<sup>1)</sup>Forty explants were evaluated.



**Fig. 1.** Primary somatic embryogenesis from cotyledon of zygotic embryo of *Panax ginseng*. (A) Indirect primary somatic embryogenesis from zygotic embryo (ZE) on MS medium containing 2,4-D (1 mg/L) and kinetin (0.1mg/L). (B) Magnified image from (A), dotted line indicates multiple primary somatic embryo (PE). (C) Direct primary somatic embryogenesis from ZE on MS basal medium. (D) Magnified image from (C), dotted line indicates the magnified PEs. (E) Maintained culture of primary somatic embryogenic calli in flask. (F) Mass production of primary somatic embryogenic calli in bioreactor. R, root.

they turned out to be mostly multiple embryos, from which quality roots could not be obtained (Fig. 1A, B; Table 1), whereas direct somatic embryogenesis was occurred on hormone-free MS basal medium (Fig. 1C, D).

### Maintenance of embryo

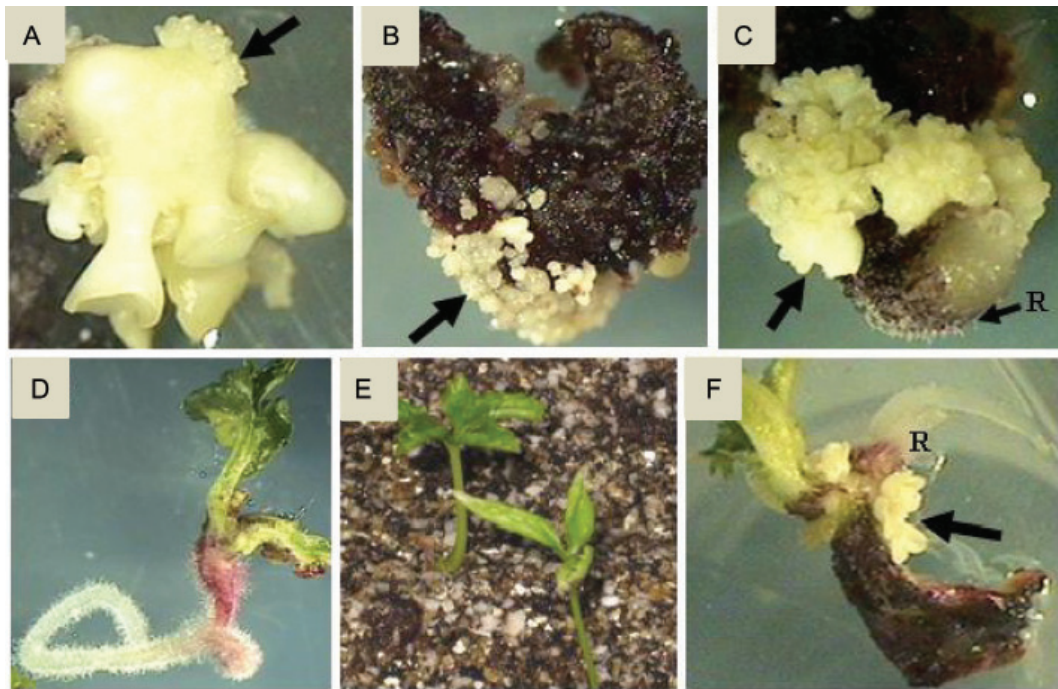
The primary somatic embryos cultured on EM media were kept at the cotyledonary stage as long as they were not detached from the parent embryos, whereas somatic

embryos on MS media developed leafy shoots although they were still attached to the parent embryo. Based on this feature, the somatic embryos were possibly can be proliferated continuously for years through subculture of embryogenic callus repetitively (Fig. 1E). Mass production was also possible by means of bioreactor culture (Fig. 1F). Total fresh weight during embryogenic cell proliferation increased approximately 260% (26 g) after 4 wk of 1.5 liter culture, whereas increased 34 % (6 g) of

**Table 2.** Increased rate of fresh weight in somatic embryogenic callus culture on EM media

	Fresh weight of somatic embryo clumps (g)		Weight increase after 4 wk (%)
	Initial inoculum	After 4 wk	
40 mL culture in 100 mL flask	4.5±0.5	6.0±1.1	34±8
1.5 L culture in 2 L bioreactor	7.3±0.6	26.0±3.8	260±30

The values represent the mean±standard error.



**Fig. 2.** Secondary somatic embryogenesis from primary somatic embryos (PEs) in *Panax ginseng*. Secondary somatic embryos (SEs, indicated by arrow) formed from the pre-cotyledonary embryo (A), cotyledonary embryo state (B), and from plantlet state (C) of a PE on hormone-free MS medium. (D) Regenerated SE plantlet on EM media. (E) Well-developed plantlets on pot. (F) Further somatic embryogenesis from the previous primary embryo. R, root.

40 mL culture (Table 2). This culture system can be applied for the maintenance of somatic embryogenic callus such as medicinal raw materials for long periods without losing any embryogenic competency since most of the somatic embryogenic calli were able to follow secondary embryogenesis.

#### Induction of the secondary somatic embryos and optimal growth stage of explants

Secondary somatic embryogenesis usually occurs when primary embryos fail to mature to plantlets, and instead give rise to successive cycles of embryos, most commonly from the cotyledons or hypocotyls [18]. In *P. ginseng*, secondary somatic embryos originated directly from the primary embryos on hormone-free MS medium within 2 wk of culture, and proliferated further

in a cyclic manner. They were visible from the embryo and cotyledon in (pre)-cotyledonary embryo state, and from the hypocotyl and root pole in plantlet state of the primary embryos (Fig. 2A-C). Among two tested media strength, we observed MS basal medium produced over 50 embryos per explants (average 30 to 40 tested) with 88% of efficiency for secondary embryogenesis, whereas EM media produced 20 to 50 embryos per explants with 57% of efficiency. This result was expected based on the previous report that high concentration of ammonium ion stimulated the frequency of somatic embryo formation [19].

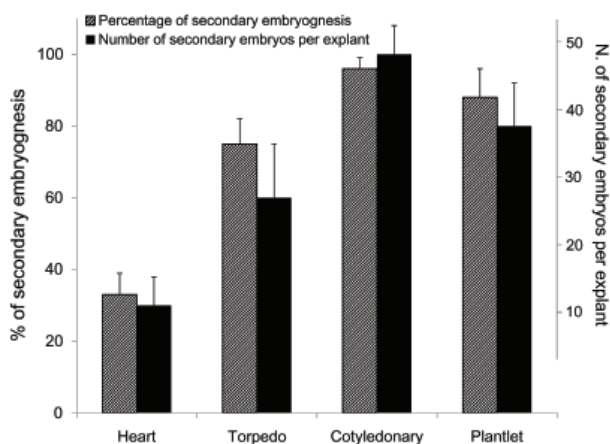
Influence of the developmental stage of explants for the secondary embryogenesis was tested. As shown in Fig. 3, more SEs were produced from cotyledonary embryos than from pre-cotyledonary shaped embryos.

**Table 3.** Effect of medium strength and sucrose concentration for the secondary somatic embryos maturation and germination

MS medium strength	Sucrose conc (%)	AC conc (%)	Speed of maturation from globular stage to germination (wk)	Germinated embryos (%)
1/3 of modified MS <sup>1)</sup>	2.0	0	Grows fast (3-8)	88
	3.0	0	Grows slowly (12-16)	45
Modified MS	2.0	0	Grows slowly (12-16)	67
	3.0	1.0	Grows very slowly and turns brown (stop growing)	0
MS	3.0	0.5	Grows slowly (12-16)	24
	3.0	1.0	Grows very slowly and turns brown (stop growing)	0

MS, Murashige and Skoog; AC, activated charcoal; conc, concentration.

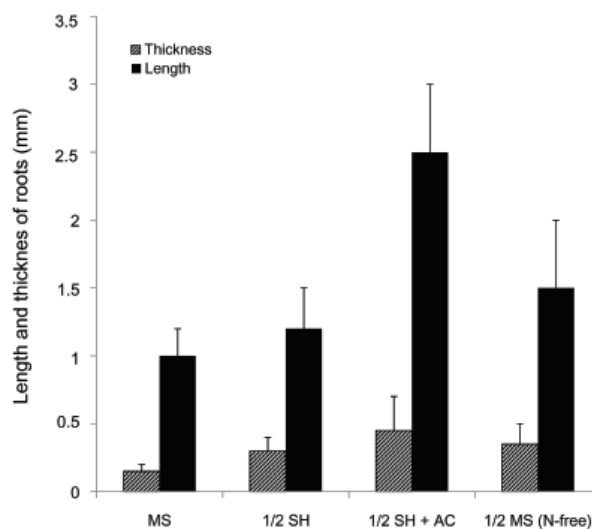
<sup>1)</sup>Modified MS contains half amount of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub> of MS.



**Fig. 3.** Influence of the developmental stage of primary somatic embryos of cv. Yunpoong in the formation of secondary somatic embryos after 30 d of culture on hormone-free MS medium.

### Maturation of secondary somatic embryos and its germination condition

Although some species do not require additional culture steps for maturation of embryos, the development of many other species is stopped at the globular stage [6]. Thus conversion from the globular stage to further stage is essential to obtain plantlet. In our investigation, reduced sucrose concentration and nitrate enhanced SEs maturation as well as conversion to plantlet in *P. ginseng*. In case of many species, increasing sucrose concentration improved embryo maturation [6,7,12]. However transfer from the globular embryo stage to the next stage was accelerated at lower sucrose concentration from 3% to 2% in all tested medium conditions and the germination ratio was also high (Table 3). In a low concentration of nitrate salts of potassium and ammonium nitrate, SEs maturation was more intensive. Addition of activated charcoal (AC) resulted in inhibition of SEs maturation (Table 3). When SEs obtained from PEs were transferred onto EM (one third of modified MS medium [MS medium containing half amount of NH<sub>4</sub>NO<sub>3</sub> and KNO<sub>3</sub>] with 2%



**Fig. 4.** Effect of basal medium for the development of plantlet roots of *Panax ginseng*, after 6 wk of culture. Murashige and Skoog (MS) and Schenk and Hildebrandt (SH) basal media were tested with and without activated charcoal (AC, 0.5%) and ammonium nitrate (N-free).

sucrose) medium, maturation and germination of SEs rapidly occurred within 2 mo.

### Plantlet development

Germinated SEs on EM medium were developed to plantlets with in average 2 cm of root-length (Fig. 2D). It was reported that the high level of ammonium nitrate in the nutrient medium is detrimental to root development in carrot [20]. In order to test the amount of nitrate in ginseng, two different-type of media with or without AC and nitrate were used for the development of plantlet roots (Fig. 4). On basal MS medium they became brown and did not show apparent visible taproots. SH medium with AC increased the root length and thickness the most among tested media conditions. It was even better than MS media without ammonium nitrate. All plantlets derived from the SEs were acclimatized and successfully transferred to soil mixture (Fig. 2E). If they were on the

same medium more than 3 mo, further secondary somatic embryogenesis was emerged from the root pole of previous plantlet (Fig. 2F).

## DISCUSSION

Secondary somatic embryogenesis is the basis of scaling up of somatic embryogenesis, and it can be utilized for the mass propagation and genetic transformation. The ratio of embryo production was higher from the secondary embryogenesis rather than from the primary embryogenesis in *P. ginseng*, which is similar to other plant species [6]. Primary embryos were obtained in ratio of 32.5% and 45% respectively from without and with hormone (Table 1), whereas higher rate (88%) of embryo explants initiated secondary embryogenesis from the all obtained primary embryos. Secondary embryos were originated directly within 2 wk of culture from the primary embryos, and they can be obtained more from the multiple primary embryos. Production of multiple primary embryos can be achieved by culturing explants on media containing hormones such as 2,4-D and kinetin (Table 1). This result suggests that the large-scale propagation of ginseng plant is possible via enhanced secondary embryogenesis from multiple primary embryos.

Primary embryos are originated from excised cotyledons of zygotic embryo. However once they are excised, they did not generate secondary embryo. Secondary embryogenesis mostly occurs from the regions such as hypocotyls and root pole of PEs (Fig. 2). In the gymnosperm species, young stages consisting of pre-embryogenic masses or pre-globular embryos are subjected to secondary embryogenesis. Otherwise, in most angiosperm dicot species, a wider range of explants can be used for secondary embryogenesis [6]. In case of *P. ginseng*, cotyledonary PEs showed the highest formation of SEs among the various developmental stages of PEs (Fig. 3). In *Acatopanax senticosus* [21], *Euphoria longan* [22], *Juglans nigra* [23], and *Manihot esculenta* [11], mature embryos are used for secondary embryogenesis. In *Brassica napus* [24] and *M. sativa* [25], embryo derived plants were even subjected to this process.

Maturation of embryo through successive developmental stages during somatic embryogenesis is important by overcoming globular stage [6] especially in case of ginseng plant. Reducing sucrose concentration and nitrate turned out to be beneficial for the enhanced secondary embryo maturation as well as conversion to plantlets (Table 3). This result is somewhat distinct compared to that of other species, where higher concentration of

sucrose gave rise to more secondary embryo maturation [6,7,12]. Sucrose concentration in the medium influenced the proliferation of secondary embryos as well as embryo differentiation into various developmental stages. Sucrose at 2% concentration stimulated the development of globular embryos to further advanced stages. Sucrose at 3% concentration stimulated the proliferation but restricted the development of somatic embryos. In case of *E. longan*, somatic embryo maturation also improved at lower concentration of sucrose [22]. We observed that less than 20 to 50 embryos per explants were produced on half ammonium and nitrates-containing MS medium, whereas over 50 embryos on intact MS, which are similar to the previously reported result [19]. Namely, lowering concentration of ammonium and nitrates promoted development of somatic embryos (Table 3), however higher concentration stimulated the frequency of somatic embryo formation in ginseng. AC is used to remove phytohormone auxin from the developing embryo. The addition of AC can enhance the conversion of somatic embryo to plantlets during maturation of somatic embryos [26,27]. It was also reported that AC caused detrimental effect such adsorption of various medium components which are inhibitory to the growth or development [28,29]. Exogenous addition of AC in ginseng seems also absorb materials required for SEs maturation, and resulted in lowering embryo maturation (Table 3). Ginseng plantlets can be obtained easily within 2 mo when secondary embryos derived from the primary embryos were detached and kept on EM medium following rapid maturation and germination of SEs. In our experience, torpedo stage of somatic embryos improved the most of conversion to plantlets, which is the similar case with *M. sativa* and *M. truncatula* [9,25].

Taken together, high efficiency of induction, germination and conversion of secondary embryos as well as establishment of regenerated plantlets were studied in this present study. High proliferation and regeneration rate of SEs in '*P. ginseng*' are very useful for genetic improvement of this type of medicinal plant. Continuous proliferation of somatic embryos via secondary embryogenesis is both cost and time effective, and is independent of the explant source. Therefore, this useful system can be applied for the micropropagation and for future genetic transformation of ginseng plant.

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