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Optimization of Direct Somatic Embryogenesis from Mature Zygotic Embryos of *Panax ginseng* C. A. Meyer

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Culture conditions were optimized for somatic embryogenesis of *Panax ginseng*. The highest frequency of embryo formation was obtained when tissues were excised from the middle region of the cotyledon segments of zygotic embryos. Only treatment with light could stimulate the formation of single-type somatic embryos, whereas multiple-type somatic embryos and calli were observed under dark conditions. The highest production of somatic embryos was found with an NH_4^+:NO_3^- ratio of 21:39. Among the tested media (MS, B5, and SH), maximum formation of somatic embryos was obtained when cotyledon explants were cultured on an 1% agar MS medium supplemented with 5% sucrose. Regenerated ginseng plantlets were transferred to an autoclaved soil mixture in the greenhouse. These transformants showed no detectable variations in their morphology or growth characteristics compared with the donor plant.

Keywords: agar, macro salts, *Panax ginseng*, somatic embryogenesis, sucrose

Panax ginseng C. A. Meyer is an important pharmaceutical plant that has been widely used as a traditional medicine since ancient times. Its cultivation is difficult in the field because shade conditions must be maintained over the four to six years that are required before the roots can be harvested. Furthermore, root rot disease precludes future cultivation of this crop in the same soil (Cho et al., 1999).

These problems demonstrate why it is important to breed ginseng to improve its genetic characters. Although wild ginseng plants are a valuable source for such programs, their over-exploitation worldwide has made them very scarce. Conventional breeding is difficult and impractical because of the lack of conserved germplasm and the lengthy culturing procedure, longer than 50 years. Therefore, biotechnological applications may provide alternative approaches for the breeding, propagation, and production of raw materials for medicinal use.

Regeneration of *P. ginseng* has been attempted via tissue culture, using somatic embryogenesis (Lee et al., 1989; Arya et al., 1991; Cellarova et al., 1992; Ahn et al., 1996; Choi et al., 1998a; Han et al., 2006). However, most of those previously regenerated plants could not be maintained when moved to soil. Only Choi et al. (1998b) have reported the successful transfer of regenerated plants derived from direct somatic embryos induced from cotyledon explants on a hormone-free medium. That regeneration protocol has proven very efficient for the induction of whole plantlets. However, although we have previously investigated the effect of macro salt stress on somatic embryogenesis without the elimination of the ginseng embryo axis (Choi et al., 1998a), the composition of the media with respect to nitrogen source and agar type had not yet been systemically optimized.

Here, we studied how the regeneration efficiency of mature ginseng embryo-base cultures could be enhanced by optimizing the nitrogen composition and the concentrations of sugar and agar in the culture media. We also examined how the performance from various explant regions could be manipulated by inoculation and lighting conditions.

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MATERIALS AND METHODS

Plant Material

Stratified seeds of *P. ginseng* were obtained from the Division of Ginseng and Medicinal Crops at the National Institute of Crop Science (Korea). For the explant source, we used fully mature, cotyledonary-stage zygotic embryos, as described by Choi et al. (1998b). Seeds were immersed in 70% ethanol, then sterilized for 15 min with a 3% sodium hypochlorite solution containing 0.1% Tween 20 before being rinsed five times with sterile distilled water. After carefully dissecting the zygotic embryos, we placed the abaxial sides of the excised cotyledons on the surface of the test medium. These media were adjusted to pH 5.8 and autoclaved at 121°C for 15 min. The culture room was maintained at 23 ± 2°C, under a 16-h photoperiod (24 μmol m^{-2} s^{-1} from white-fluorescent tubes).

Explant Regions of Zygotic Embryos

To determine which explant type was most suitable for producing somatic embryos, the zygotic embryos were excised transversely into three pieces (Fig. 1), then cultured on an MS basal medium (Murashige and Skoog, 1962; 3% sucrose, 0.8% agar). After two months, we calculated the frequency of somatic embryo formation and counted the number of embryos per explant.

Media Composition

Cotyledon explants (10 per Petri dish, 30 per treatment) were cultured on one of three types of media: MS, B5

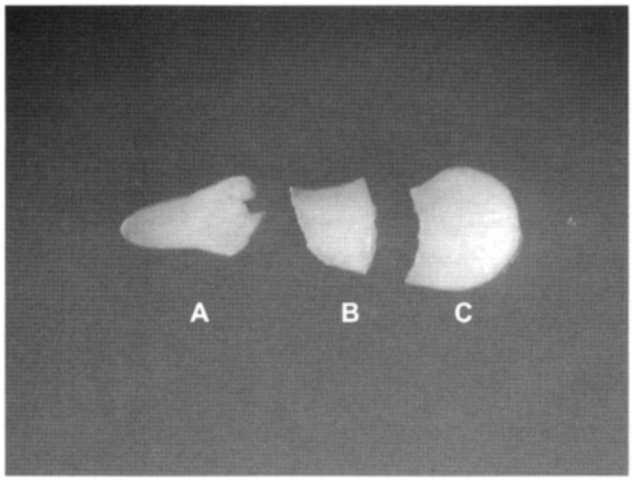

Figure 1. Three regions of Korean ginseng zygotic embryos as explant sources. Total length of 6 mm, with regions **A**, **B** and **C** being about 2.2, 1.8, and 2 mm, respectively.

(Gamborg et al., 1968), or SH (Schenk and Hildebrandt, 1972). To optimize for the MS media, we also tested five ratios of NH_4^+ and NO_3^- (0:60, 21:39, 30:30, 39:21, or 60:0 mM). These ratios involved KNO_3, NH_4NO_3, NH_4Cl, or $NaNO_3$. KCl replaced the KNO_3 in order to maintain the original K^+ concentration of the basal medium. After two months of culture, we determined the frequency of somatic embryo formation and number of embryos per explant. These experiments were repeated five times.

Optimization of Sugar and Agar Concentrations

To examine the influence of sucrose level on direct somatic embryo formation, mature embryo explants were cultured on an MS medium containing 1% agar, under illumination. Sucrose concentrations were adjusted to 1, 3, 5, 7, or 9% before autoclaving. For the optimization of agar concentration, explants were cultured on an MS medium containing 5% sucrose, with an agar concentration of 0.5, 0.8, 1.0, or 1.5%. In all cases, the frequency of somatic embryo production was determined after two months of culture.

Germination and Plant Regeneration

Germination was induced by transferring the cotyledon explants together with the cotyledonary somatic embryos to a 1/2 MS medium containing 5 mg L^{-1} GA_3 and 3% sucrose for two to four weeks. The partially germinated embryos were then separated from their parent explants by forceps, and consecutively sub-cultured on the same medium type but without GA_3. When their shoots grew to 7-cm long, the rooted plantlets were moved to the greenhouse, where they were grown in pots containing a 1:1:1 mixture (by volume) of autoclaved soil, sand, and peat, as described by Choi et al. (1998b).

Statistical Analysis

Statistical calculations were carried out according to the analysis of variance (ANOVA), and the results were examined according to Duncan's multiple range test (Duncan, 1955). In all cases, values represented the means of five replicate Petri dishes per treatment.

Table 1. Frequency of somatic single-embryo formation from three types of *P. ginseng* cotyledon explants after 2 months of culture on a hormone-free MS medium.

Explant region[a]	Somatic embryo production (%)[b]	No. of somatic embryos/explant[b]
A	0.0c	0.0c
B	65.4a	22.1a
C	0.52b	2.5b

[a]A, B, and C regions of explant are described with Figure 1.
[b]Means not followed by the same letter within a column are significantly different at the 5% level, by Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Inoculation Regions

In *P. ginseng*, the cotyledon explants of zygotic embryos can produce somatic embryos on a hormone-free medium (Choi and Soh, 1997). Here, we examined which portion of a zygotic embryo was most suitable for use as an explant source in order to optimize the formation frequency. Zygotic embryos were transversely cut into three pieces (Fig. 1), which were then cultured on an MS medium with 3% sucrose and 0.8% agar. Induction of direct somatic embryos occurred at the highest frequency when explants were taken from the middle region of the cotyledon (Region B), while little or no formation was obtained from Region A or C, respectively (Table 1). This indicates that the potential for producing somatic embryos differs by explant source. Choi and Soh (1996) also have demonstrated that both the plumule and the radicle tip of the embryo axis contain water-diffusible substances that inhibit somatic embryo development, thus making it necessary to detach the cotyledon explants from that axis to induce somatic embryo formation.

Lighting Conditions

Cotyledon explants were cultured for two months under either darkness or illumination on MS media containing 3% sucrose and 0.8% agar. Although the frequency of somatic embryo formation did not differ significantly between these light and dark conditions, their morphologies did (Table 2). For example, somatic embryos either developed into separate, single-type embryos, or else fused with other embryos or explants, resulting in multiple-type embryos. The former was induced only under light conditions, whereas calli and the multiple-type somatic embryos were formed mainly under darkness (Fig. 2A, B). Choi et al. (1998b) have previ-

Table 2. Effects of illumination on the formation of single embryos from *P. ginseng* cotyledon explants after 2 months of culture on a hormone-free MS medium.

Lighting condition	Somatic embryo production (%)[a]	No. of somatic embryos/explant[a]	
		Single	Multiple
Light	68.2a	20.3a	2.7b
Dark	65.0a	0.0b	13.1a

[a]Means not followed by the same letter within a column are significantly different at the 5% level, by Duncan's Multiple Range Test.

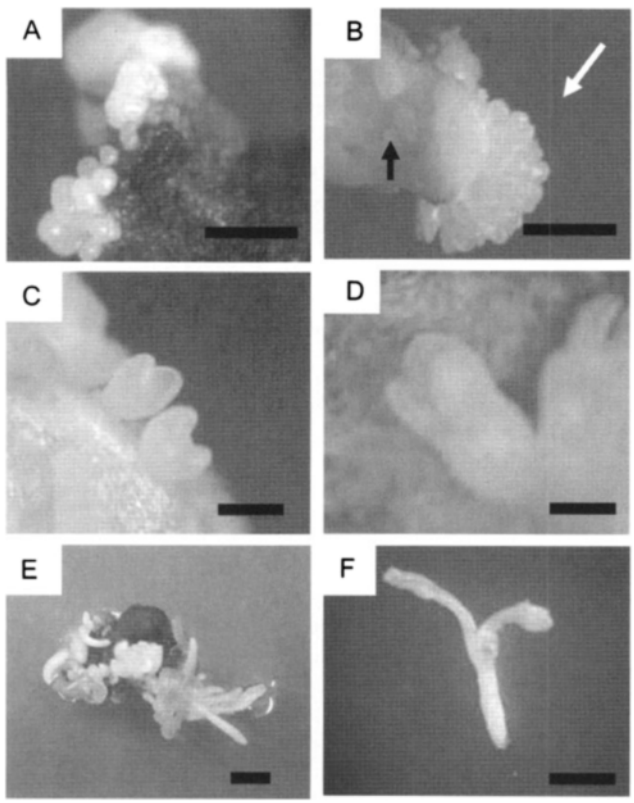

Figure 2. Plant regeneration via direct somatic embryogenesis of *P. ginseng*. **A**, Single-type somatic embryos formed on surface of mature zygotic embryo; **B**, Multiple-type somatic embryos induced under the dark condition. Black and white arrows indicate callus and multiple-type somatic embryos, respectively; **C**, Heart stage of somatic embryo; **D**, Torpedo stage of somatic embryo; **E**, Germination of somatic embryos with 5 mg L^{-1} GA_3; **F**, Rooting induction for regenerated plantlet. Bars represent 1.0 mm (**A**), 3.0 mm (**B**), 0.5 mm (**C**), 0.3 mm (**D**), 7.0 mm (**E**), and 15.0 mm (**F**).

ously detailed these two types of embryo formation, and have reported that this difference is determined by the number of cells that participate in this somatic embryo production. In the current study, illumination caused the development of single-type somatic embryos to progress through the globular, heart-shaped, torpedo-shaped, and cotyledonary stages, as is typical for zygotic embryos (Fig. 2C, D), suggesting that light affects the cell-cell interrelationship, eliciting such different types of somatic embryo formation.

Media Composition

Cotyledon explants cultured on MS, B5, or SH media showed important differences in their somatic embryo development. The highest frequency of embryo formation (23.2) was obtained on the MS medium (Table 3). This variation was primarily due to the amount of ammonium supplied by their respective nitrogen sources, with the MS medium containing the greatest concentration. This suggests that ammonium might stimulate the formation of somatic embryos from mature embryo explants of ginseng, as was also demonstrated earlier (Choi et al., 1998a). Because the nitrogen composition of the culture medium clearly affects embryogenesis and plant regeneration (Fisichella et al., 2000; Nuutila et al., 2000), we also investigated

Table 3. Effects of media and NH_4^+:NO_3^- ratios on *P. ginseng* single-embryo formation after 2 months of culture.

Media and macro-element	Treatment	Somatic embryo production (%)[a]	No. of somatic embryos/explant[a]
Media	MS	69.1a	23.2a
	B5	35.6b	7.8b
	SH	21.3c	3.5c
NH_4^+:NO_3^- ratios of MS medium	0:60	0.0c	0.0d
	21:39	65.2a	15.8a
	30:30	60.5ab	9.2b
	39:21	50.4b	6.5c
	60:0	0.0c	0.0d

[a]Means not followed by the same letter within a column are significantly different at the 5% level, by Duncan's Multiple Range Test.

how the ratios of NH_4^+ (A) to NO_3^- (N) might influence the induction of somatic embryos from cotyledon explants (Table 3). Here, the highest frequency of formation was observed at an A:N ratio of 21:39, which is consistent with the standard concentration required for an MS basal medium.

When the culture medium contained only NH_4^+, no somatic embryos developed and the explants became necrotic or died. Compared with other salts, NH_4NO_3 has been shown to be the most important factor for somatic embryo formation (Choi et al., 2003). Likewise, the number of cyclamen somatic embryos increases when anther cultures are treated at a concentration of 20 mM NH_4^+ and 10 mM NO_3 , or ovary cultures are exposed to 10 mM NH_4^+ and 20 mM NO_3^- (Kiviharju et al., 1992). In pepper (*Capsicum annuum*), a concentration of 40 mM NH_4^+ and 20 mM NO_3^- causes a decline in the number of somatic embryos produced (Buyukalaca and Mavituna, 1996).

Optimization of Sucrose and Agar Concentrations

Glucose, mannose, fructose, and sorbose can be used to support somatic embryogenesis and organogenesis in calli derived from the cotyledons of mature ginseng zygotic embryos (Tang, 2000). Although the best regeneration of somatic embryos is obtained on a medium containing glucose as the sole carbon source, the concentration of sucrose had not previously been optimized in the early stage of

Table 4. Frequency of somatic single-embryo formation from cotyledon explants of *P. ginseng* on an 1% agar MS medium supplemented with different sucrose concentrations.

Sucrose concentration	Somatic embryo production (%)[a]	No. of somatic embryos/explant[a]
1%	0.0d	0.0d
3%	71.1a	22.3ab
5%	73.8a	26.7a
7%	57.5b	5.3c
9%	10.9c	0.0d

[a]Means not followed by the same letter within a column are significantly different at the 5% level, by Duncan's Multiple Range Test.

Table 5. Frequency of somatic single-embryo formation from cotyledon explants of *P. ginseng* on an MS medium containing 5% sucrose and different agar concentrations.

Agar concentration	Somatic embryo production (%)[a]	No. of somatic embryos/explant[a]
0.5%	70.2b	8c
0.8%	69.3b	12b
1.0%	75.5a	25.3a
1.5%	0.0c	0.0d

[a]Means not followed by the same letter within a column are significantly different at the 5% level, by Duncan's Multiple Range Test.

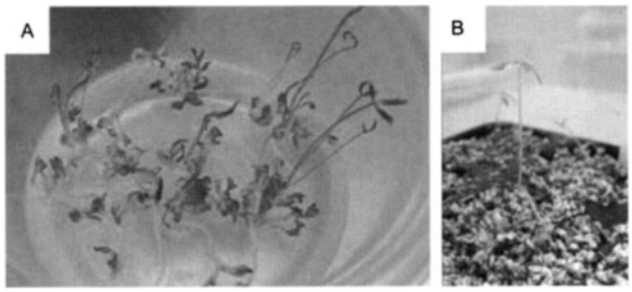

Figure 3. Transfer of *P. ginseng* plantlets to greenhouse. **A**, Whole plant obtained from 1/2-strength MS medium; **B**, Acclimatized plantlets regenerated from single embryos at 2 months after transfer.

inoculation. However, Choi and Soh (1997) have reported that somatic embryo formation in ginseng is enhanced by plasmolyzing treatment with sucrose. To investigate the effect of sugar on the formation of single somatic embryos, we applied 1, 3, 5, 7, or 9% sucrose to MS media containing 1% agar, and found that the ideal level was 5% (Table 4). Although the frequency of somatic embryo formation did not differ significantly between 3 and 5%, the number of somatic embryos induced from explants was higher at the latter concentration. Furthermore, embryo production decreased when more than 7% sucrose was used, whereas necrosis of the explants occurred with 1% sucrose.

We also examined the effect of agar strength in the MS media. Somatic embryo production was superior in 1% agar supplemented with 5% sucrose; cotyledon explants did not respond when the medium contained 1.5% agar (Table 5). Klimaszewska et al. (2000) have investigated the influence of gel strength on embryogenesis in eastern white pine, and have found that a high agar concentration is associated with the reduced availability of water to the explants, proving to be the most important factor in enhancing the embryogenic response.

In our final experiments to induce germination, somatic embryos were transferred to a 1/2-strength MS medium with 5 mg L^{-1} GA_3, and were cultured until the shoots were 1.0- to 1.5-cm long (Fig. 2E). After the germinated embryos were separated from their explants, they began to produce roots following three weeks of culture on a 1/2 MS medium without GA_3 (Fig. 2F). The regenerated ginseng plantlets (7-cm tall) were then transferred to an autoclaved soil mixture and reared in a greenhouse (Fig. 3A, B). In all, 80% of the plantlets survived without wilting or chlorosis at two months after this transfer. These transformants showed no obvious variations in their morphology or growth characteristics compared with the donor plant.

In conclusion, we report here the optimization of culture medium, NH_4^+/NO_3^- ratio, and sucrose and agar concentrations for the formation of somatic embryos from ginseng. In these experiments, the highest frequency of embryo production from cotyledon explants was obtained on an 1% agar MS medium supplemented with 5% sucrose.

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