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Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormone-free medium

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Abstract Cotyledon explants of Korean ginseng (*Panax ginseng* C. A. Meyer) produced somatic embryos directly on growth regulator-free medium. Somatic embryos developed as either multiple or single-state forms, depending on the degree of maturity of the cotyledons. Cotyledon explants from midmature zygotic embryos formed multiple embryos, while cotyledons from fully mature zygotic embryos formed single embryos. Somatic single embryos regenerated into normal plantlets with both roots and shoots, while multiple embryos did not produce roots but regenerated only into multiple shoots. In full-strength MS basal medium, the root growth of plantlets derived from single embryos was weak compared to that of shoots. Deletion of ammonium nitrate from the MS medium promoted the root growth of the plantlets. The ginseng plants with well-developed shoots and roots regenerated from single embryos were successfully acclimatized in a greenhouse when they were planted in soil.

Key words Direct somatic embryogenesis · *Panax ginseng* · Plant regeneration · Propagation

Introduction

Korean ginseng plants (*Panax ginseng* C. A. Meyer) are perennial herbaceous plants which grow very slowly. A cultivation period of more than 3 years is required before the plants produce seeds. At the time of seed harvest, zygotic embryos of Korean ginseng are still in an immature globular stage, thus the seeds require stratification and cold treatment for several months. Under natural conditions, germination is obtained after 21 months of maturation. Therefore, tissue culture procedures could contribute to clonal propagation and breeding in ginseng. However, it has been accepted that the regeneration of plants with well-developed roots from somatic embryos of ginseng is a very recalcitrant process. In many investigations on Korean ginseng tissue cultures, structurally abnormal somatic embryos such as multicotyledonary or multiple embryos were frequently formed (Butenko et al. 1968; Chang and Hsing 1980; Lee et al. 1990; Arya et al. 1991, 1993). In addition, the regenerated plantlets from somatic embryos developed into multiple shoots without or inadequate roots (Chang and Hsing 1980; Shoyama et al. 1988; Cellarova et al. 1992). Root development in plants is indispensable for field habituation after transfer. However, little is known on why ginseng plants do not form well-developed roots. In addition, there has been no report on the successful field transfer of Korean ginseng plants regenerated from somatic embryos.

In general, exogenous growth regulators are the most important components for somatic embryo induction. In *Panax ginseng*, the majority of investigations on somatic embryogenesis were conducted from callus culture (Butenko et al. 1968; Chang and Hsing 1980; Lee et al. 1990; Arya et al. 1991, 1993). However, cotyledon explants of *Panax ginseng* can produce somatic embryos directly on medium without any growth regulators (Harn and Lee 1974; Lee et al. 1990; Choi and Soh 1994). Choi and Soh (1996; Choi et al. 1997) revealed that direct somatic embryo formation from ginseng zygotic embryos on regulator-free medium is related to the excision of zygotic embryos and polar endogenous auxin accumulation. The regeneration of *Panax ginseng* plants through direct somatic embryogenesis on growth regulator-free medium has not been reported.

The present paper deals with the regenerative ability of somatic multiple and single embryos arising directly from ginseng cotyledons on medium without any growth regulators and the high frequency of normal plant regeneration under field conditions.

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Materials and methods

Somatic embryogenesis

Korean ginseng (*Panax ginseng* C. A. Meyer) seeds were harvested from the experimental garden of the Korea Ginseng and Tobacco Research Institute. The seeds were stratified in humidified sand to mature for several months at a 10°C since the zygotic embryos just after harvest were in an immature globular stage (at about 200 μm in length). During stratification, developing zygotic embryos at four stages of maturity were sampled at various times for experimentation: immature cotyledonary (1 mm in length), midmature cotyledonary (3 mm in length), fully mature cotyledonary stage (6 mm in length) and seedling stage (60 mm in length) as represented by Fig. 1.

The seeds were immersed in 70% ethanol for 1 min, then in 1% sodium hypochlorite solution for 1 h and then washed three times with sterile distilled water. After carefully dissecting the zygotic embryos from the seeds, we placed the abaxial sides of the excised cotyledons on the surface of MS basal medium (Murashige and Skoog 1962) with 5% sucrose, 0.7% agar. The medium was adjusted to pH 5.8 and then autoclaved at 120°C for 15 min. Cotyledon explants were cultured in 10×1-cm plastic petri dishes containing 30 ml of medium. Fifteen cotyledon explants were cultured on each petri dish. Triple replicates were used for per treatment, which was replicated three times. The culture room was maintained at 24° ± 2°C under 16:8-h photoperiods of 24 μmol m^{-2} s^{-1} under white-fluorescent tubes. The frequency of somatic embryo production was determined after 2 months of culture by counting cultured cotyledon explants that formed somatic embryos.

Germination and plant regeneration

Germination was induced by transferring the cotyledon explants together with cotyledonary somatic embryos to MS medium containing 3×10^{-5} *M* GA_3 (gibberellic acid) and 3% sucrose for 2 weeks. The slightly germinated embryos (about 10 mm in length) were manually removed from the parent cotyledon explants by forceps (for single embryos) or a razor blade (for multiple embryos) and then cultured on half-strength MS basal medium without GA_3 for 1 month to support the continued growth into plantlets. Fifteen germinating somatic embryos were cultured on a 10×1-cm petri dishes containing 30 ml of medium. Triple replicates were used for per treatment, which was replicated three times. The culture conditions were the same as those for somatic embryo induction. After 1 month of culture, root and shoot development from single and multiple embryos was observed.

To investigate the effect of nitrogen compounds on the root and shoot growth of plantlets, we transferred germinating embryos (10 mm in length) on MS medium with 3×10^{-5} *M* GA_3 to 50 ml liquid medium with filter bridges in 100-ml glass tube. The latter medium consisted of modified MS liquid medium with 2% sucrose and various concentrations and combinations of ammonium nitrate and potassium nitrate as shown in Fig. 6. After 2 months of culture, the growth of shoots and roots was assessed. One plant was cultured per tube. About 20 explants were used per treatment, and each treatment was repeated three times.

Transfer of ginseng plants to soil

The morphologically normal plantlets with both shoots and roots (about 7 cm in length) that developed from somatic single embryos were transferred to square plastic pots containing soil, sand and peat (4:4:3 v/v) in a greenhouse. The plants were covered with glass beakers for 3 weeks, and the then-acclimatized plants were laid open to greenhouse conditions without cover. Seven weeks after planting, the survival rate of plants was observed. Twenty plants were planted in soil, which repeated three times.

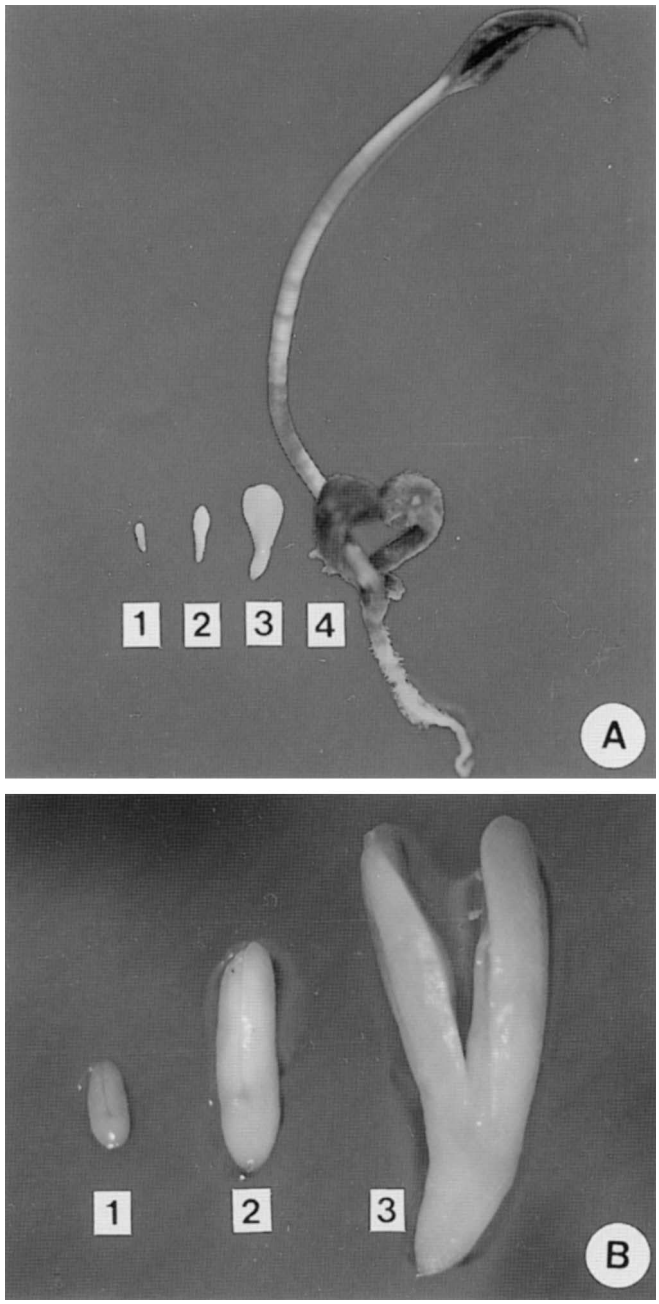

Fig. 1A, B Various stages of ginseng zygotic embryos as explant sources. **A** An immature cotyledonary zygotic embryo 1 mm in length (*1*), a midmature zygotic embryo 3 mm in length (*2*), a mature zygotic embryo 6 mm in length (*3*), a seedling 60 mm in length (*4*). **B** Enlarged view of **A**

Light and scanning electron microscopical observation

For histological observation of somatic embryo development, cotyledon explants with somatic embryos at an early stage were fixed in FAA (formalin, acetic acid and alcohol), dehydrated in ethyl alcohol and then embedded in paraffin. After the samples were cut into 10-μm sections with a rotary microtome, the sections were stained with hematoxylin. Some samples were fixed in 1% glutaraldehyde and dehydrated with ethyl alcohol and then dried in a critical point drier. After being coated with gold, the samples were observed by a scanning electron microscope (JSM T330A).

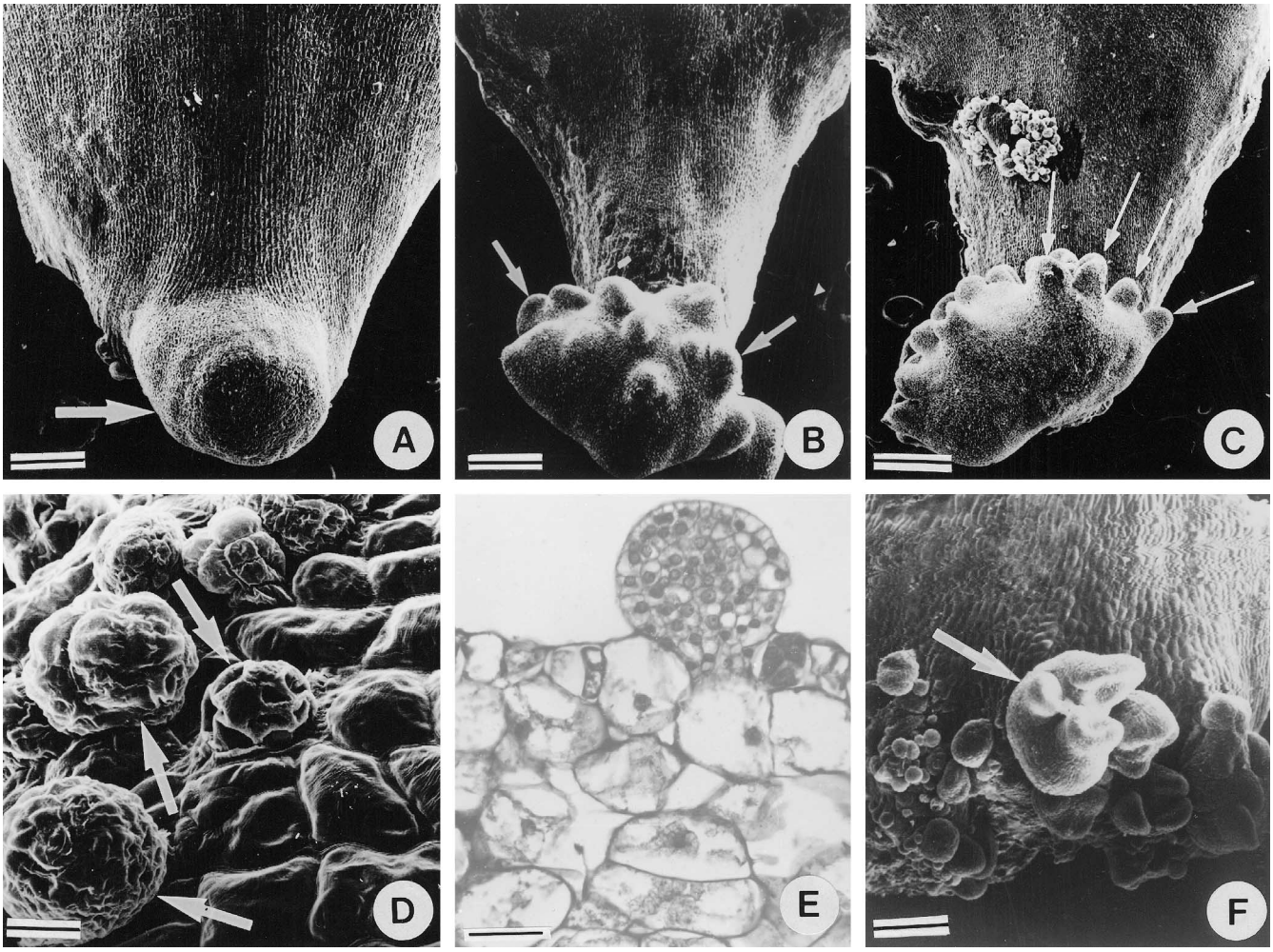

Fig. 2A–F Scanning electron and light microscopy of somatic multiple and single embryos arising directly from ginseng cotyledon explants on growth regulator-free medium. **A–C** Development of somatic multiple embryos from midmature cotyledons, **D–F** development of somatic single embryos from mature cotyledons. **A** An embryonic nodular tissue (*arrow*) formed on the basal portion of a cotyledon after 10 days (*bar*: 180 μm), **B** numerous cotyledon primordia (*arrows*) formed on a nodular tissue after 15 days (*bar*: 230 μm), **C** multiple embryos with numerous cotyledons (*arrows*) after 1 month (*bar*: 230 μm), **D** early globular somatic embryos (*arrows*) developed from single epidermal cells of a cotyledon after 10 days (*bar*: 40 μm), **E** globular embryos formed from the epidermal single cells of cotyledons after 15 days (*bar*: 120 μm), **F** cotyledonary single embryo (*arrow*) after 2 months (*bar*: 230 μm)

Table 1 Frequency of somatic multiple or single embryo formation from cotyledon explants of Korean ginseng zygotic embryos at different stages of maturity on hormone-free MS medium after 2 months of culture

Stage of explant	Size (mm)	Explants forming somatic embryos (%)[a]	Proportion of embryos	
			Multiple	Single
Immature cotyledon	1	0	0	0
Midmature cotyledon	3	94±7	96	4
Mature cotyledon	6	66±5	13	87
Seedling	60	0	0	0

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

Results

Development of multiple and single embryos

When the cotyledon explants were cultured on MS agar medium with 5% sucrose, somatic embryos developed directly near the basal excised region of the cotyledon explants (Figs. 2–4). The frequency of somatic embryo formation was the highest (93%) in cotyledon explants from midmature zygotic embryos, and the competence for embryo formation decreased to 66% as the zygotic embryos matured, with no embryos forming at the seedling stage (60 mm in length) (Table 1). Somatic embryos developed into a multiple or single state (Table 1). The frequency of multiple and single embryo formation differed markedly according to the degree of maturity of the zygotic embryos. On cotyledons from midmature zygotic embryos, most of somatic embryos (96% of total embryos) developed as mul-

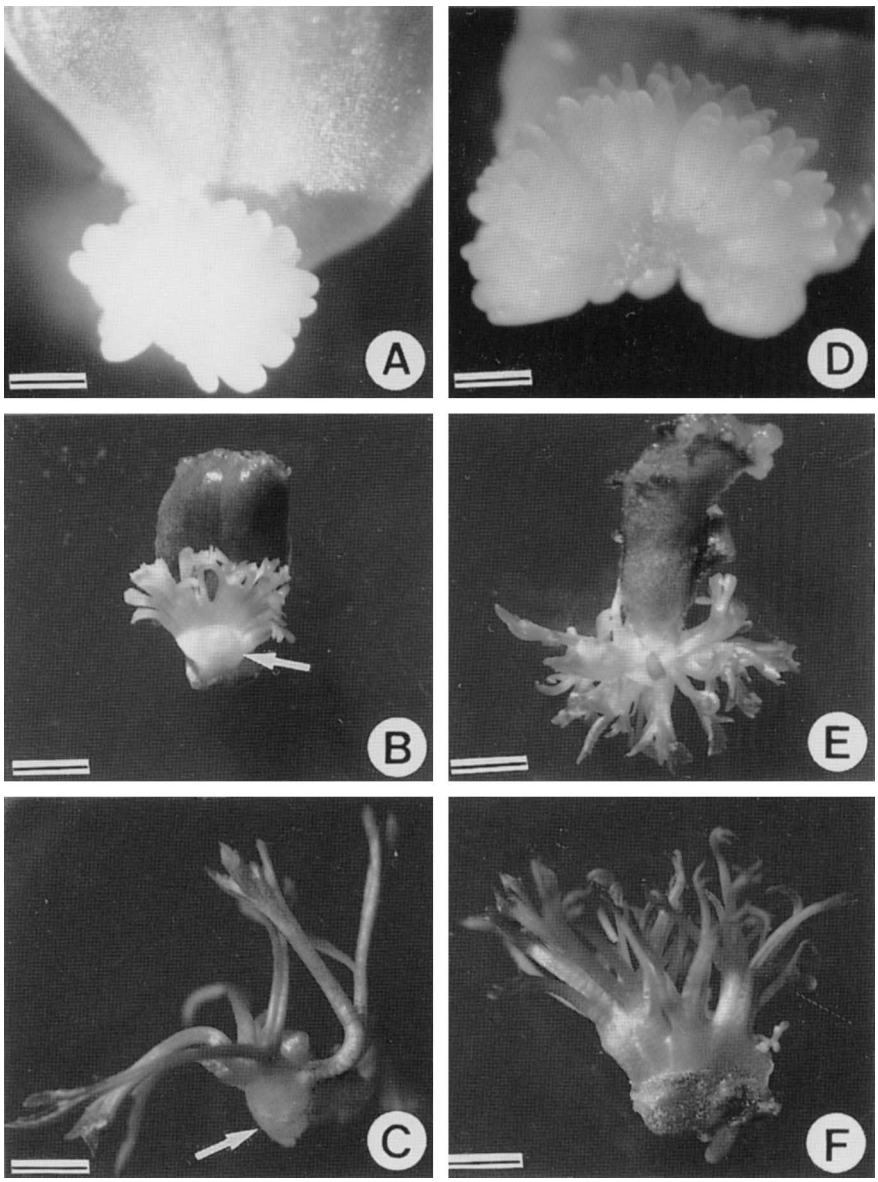

Fig. 3A–F Multiple shoot formation from somatic multiple embryos of ginseng. **A, D** Somatic multiple embryos formed directly from the basal surface of midmature cotyledons on MS basal medium after 1 month of culture (*bars*: 830 μm), **B, E** greening of cotyledonary multiple embryos just after treatment with 3×10^{-5} *M* GA_3 for 2 weeks (*bars*: 2.3 cm), **C, F** multiple shoots formed from somatic multiple embryos on half-strength MS medium (*bars*: 3.2 cm). *Arrows* in **B** and **C** indicate radicle region

tiple forms, while on cotyledon explants from mature zygotic embryos, 13% of the somatic embryos developed into single forms (Table 1). In multiple embryogenesis from midmature cotyledons, nodular tissues (Fig. 2A, arrow) formed on the basal portion of the cotyledons, these developed into numerous multiple embryos (Fig. 2B–C), and the multiple embryos fused with each other and to the parent explants (Fig. 3A, D). While, in a single embryogenesis from fully mature cotyledons, independent globular embryos formed from single epidermal cells of cotyledons (Fig. 2D, E, arrow); these eventually developed into single cotyledonary embryos (Figs. 2F; 4A, C).

Germination of embryos

When the somatic multiple and single embryos matured into the cotyledonary stage after 2 months of culture, further growth of the embryos was ceased and they showed no germination response. The color (opaque white) and structure of the somatic embryos did not change until the culture times were extended for several months. Neither multiple embryos nor single embryos germinated, even though the embryos were transferred to fresh MS basal medium without growth regulators, this might represent dormancy of somatic embryos. Upon transfer to medium with 3×10^{-5} *M* GA_3, all the somatic embryos turned green and germinated within 2 weeks of culture (Figs. 3B, E; 4C, D). The optimum concentration of GA_3 was not noticed because there were no discernible differences in color or germination from 1.5×10^{-5} *M* to 300×10^{-5} *M* GA_3 (data not presented). However, continuous culture of somatic embryos on medium with a high concentration of GA_3 (over 6×10^{-5} *M*) stimulated an abnormal slender elongation of the shoots. Thus, treatment with GA_3 was restricted to 2 weeks.

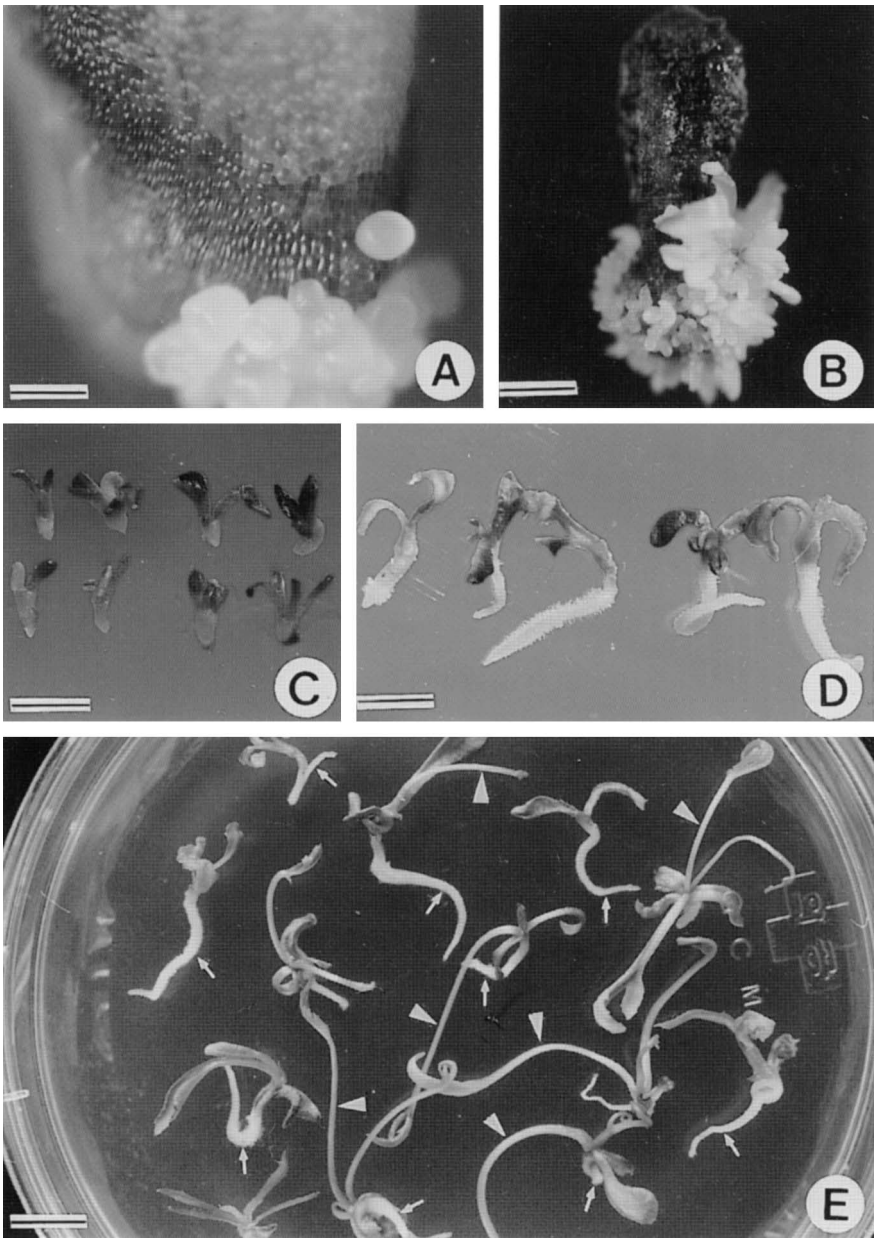

Fig. 4A–E Plant regeneration with both shoots and roots from somatic single embryos of Korean ginseng. **A** Somatic embryos formed directly from cotyledons of mature zygotic embryos on MS basal medium after 1 month (*bar*: 300 μm), **B** cotyledonary embryos after 2 months (*bar*: 920 μm), **C** somatic single embryos with well-developed cotyledons and radicles after separation from the cotyledon explants (*bar*: 1.2 cm), **D** germinating somatic single embryos with well-developed roots on MS medium with 3×10^{-5} *M* GA_3 (*bar*: 2.2 cm), **E** plantlets regenerated from single embryos on half-strength MS basal medium with both shoots and roots (*arrows* roots, *arrowheads* shoots, *bar*: 1.36 cm)

Plant regeneration of embryos

When somatic embryos were not separated from the parent cotyledon explants, embryos did not produce roots although the shoots grew well. In the culture of somatic single embryos detached individually from parent cotyledon explants, both root and shoot growth was achieved simultaneously (Fig. 4E). However, in multiple embryos (Fig. 3B, E), separation of the embryos from parent cotyledon explants was impossible without damaging the embryos because multiple embryos fused with each other and to parent cotyledon explants, and had no discernible radicles. The multiple embryos could only be separated from the parent explants by cutting the lower hypocotyl using a razor blade (Fig. 3C, F), whereas, in single embryos, the radicle tips of the embryos were gently attached to the cultured explants (Fig. 4A), and the embryos were easily separated with forceps (Fig. 4C). Of the single embryos 73% developed into normal seedlings with shoot and roots similar to those of zygotic embryos (Figs. 4E; 5). In contrast, none of multiple embryos produced roots (Figs. 3C, F; 5), even in some multiple embryos having radicle-like structures (Fig. 3B, arrow).

The seedlings (10 mm in length) that germinated from single embryos after 2 weeks of GA_3 treatment were transferred to half-strength MS basal agar medium. The proportion of root length (about 36% of total plantlet length) was still low compared to shoot length until 2 months of culture (Fig. 4E). In germinating seedlings cultured on full-strength MS medium, root growth was highly suppressed. To observe the nitrogen requirement for root growth, we transferred the seedlings derived from single embryos to

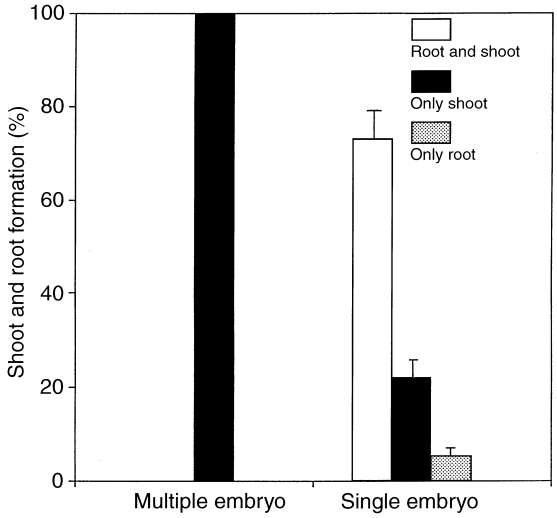

Fig. 5 Frequency of root and/or shoot growth from somatic multiple or single embryos of Korean ginseng cultured on half-strength MS basal medium. Each *bar* represents the mean SE of three independent experiments

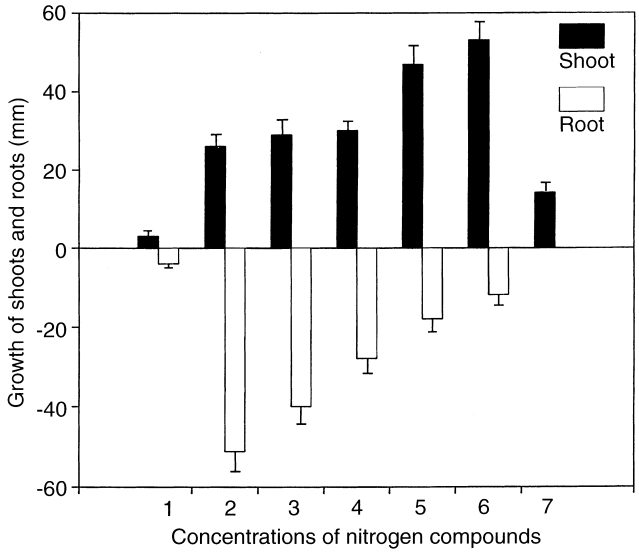

Fig. 6 Effect of ammonium nitrate and potassium nitrate on the growth of shoots and roots regenerated from somatic single embryos. *1* NH_4^+-free+NO_3^--free, *2* NH_4^+-free+4.7 m*M* NO_3^-, *3* NH_4^+-free +9.4 m*M* NO_3^-, *4* NH_4^+-free+18.8 m*M* NO_3^-, *5* 10.3 m*M* NH_4^++ 19.7 m*M* NO_3^-, *6* 20.6 m*M* NH_4^++18.8 m*M* NO_3^-, *7* 41.2 m*M* NH_4^+ +78.8 m*M* NO_3^-. Each *bar* represents the mean ±SE of three independent experiments

liquid media with a filter paper bridge in a glass tube. The nitrogen levels (ammonium nitrate and potassium nitrate) of MS medium were modified to various concentrations and combinations as shown in Fig. 6. On full-strength MS basal medium (10.3 m*M* NH_4^++19.7 m*M* NO_3^-), root growth was weak, but shoot growth was vigorous (Fig. 6). Root growth of the plants was generally vigorous in modified MS media omitting ammonium nitrate (Fig. 7A). Therefore, to promote balanced root and shoot growth, we transferred all of the germinated seedlings to half-strength MS medium lacking ammonium nitrate. In the ginseng plants regenerated from somatic embryos, the number of epicotyls (leaf) varied: 47% of the plantlets regenerated from single embryos had single epicotyls and single roots with the same morphology as those from zygotic embryos, 35.1% of the plantlets had multiple epicotyls, and 17.3% had dormant epicotyls.

Transfer of ginseng plants in soil

Ginseng plantlets (7 cm in length) having both shoots and roots derived from single embryos were transferred to a mixture of autoclaved soil [soil, sand and peat (4:4:3 v/v)] in a greenhouse. The plants were covered with glass beakers for 3 weeks and then exposed for an additional 4 weeks; 90% of the plantlets survived without wilting and the loss of their green color (Fig. 7B, C). The ginseng plants derived from somatic embryos were highly sensitive to damping-off disease resulting from fungi infection. Once fungi on the surface of the soil were visible to the eye, the leaf bases of the plants had already been infected and the plants eventually died from damping-off disease within 1–2 weeks. Twenty-seven percent of the plants survived until 15 weeks after their transfer into the greenhouse, and there are no obvious structural changes except for slightly less leaf growth compared to 7-week-old plants. In natural ginseng plants derived from seeds, the ginseng plants have small single leaves throughout the first year of cultivation. In some ginseng plants derived from somatic embryos, a new leaf developed from dormant axilary buds (Fig. 7D, arrowhead). To observe the root development of the plants, we removed the plants from the soil after 15 weeks. The main roots had thickened well, and there were many newly formed lateral roots (Fig. 7D, arrows).

Discussion

In the present experiment, somatic embryos developed into a multiple state or single state, depending on the degree of maturity of the zygotic embryos. Multiple embryos formed from midmature cotyledons were morphologically abnormal; they fused with each other and to parent cotyledon explants. In contrast, single embryos from cotyledon explants of mature zygotic embryos developed into a normal structure with a definite radicle that was closely similar to that of the zygotic embryos. In a previous paper, histological observation revealed that somatic multiple embryos always derived from the epidermal and subepidermal cell masses of ginseng cotyledons but that single somatic embryos mainly arose from epidermal single cells of cotyledons (Choi and Soh 1994). Both multiple and single embryos have no vascular connection with parental tissues. Similar morphogenesis has been reported in the culture of zygotic embryos of *Trifolium repens* (Williams and Maheswaran 1986). The multiple embryogenesis from ginseng cotyledon culture was similar to the multicellular em-

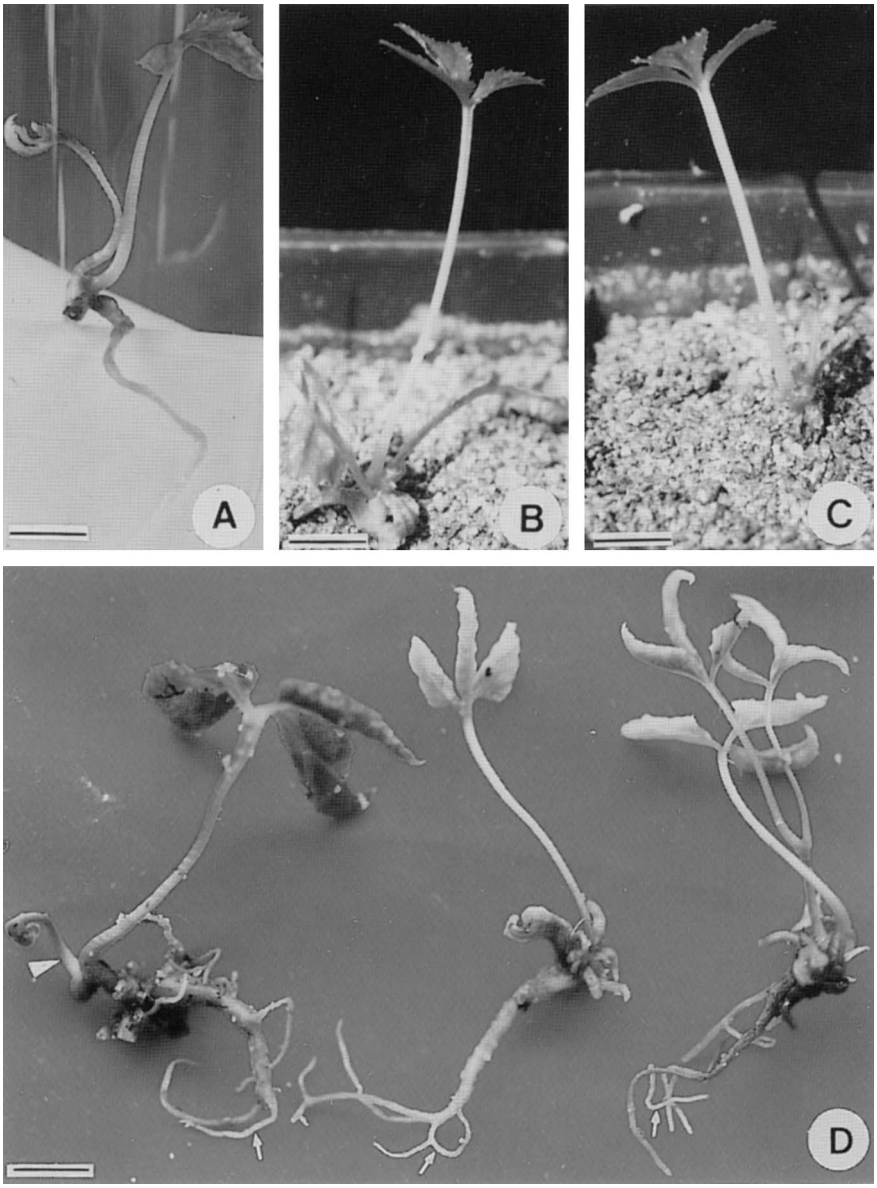

Fig. 7A–D Transfer of Korean ginseng plantlets into a greenhouse. **A** A plant with a well-developed root on half-strength MS liquid medium lacking ammonium nitrate (*bar*: 1.6 cm), **B, C** acclimatized plantlets regenerated from single embryos in a greenhouse 7 weeks after transfer (*bar*: 1.2 cm), **D** plantlets 15 weeks after transfer having well-thickened main roots and newly formed lateral roots (*arrows*) (*bar*: 1.0 cm)

bryo budding or cleavage polyembryony suggested by Williams and Maheswaran (1986).

The regenerative ability of multiple and single embryos from cultured ginseng cotyledons was observed. In multiple embryos, the detachment of embryos from cotyledon explants was impossible without damage to the embryos because the multiple embryos fused to parent explants. Even though multiple embryos could be mechanically separated from the parent explants, root production from them was not achieved. Single embryos were easily separated from the parent explants because only their narrow radicle tips were attached to the parent cotyledon explants; single embryos then regenerated into morphologically normal plants with well-developed roots and shoots. In most *Panax ginseng* tissue cultures, somatic embryos are frequently regenerated into multiple shoots that either miss a root system or have an inadequate one (Chang and Hsing 1980; Shoyama et al. 1988; Cellarova et al. 1992; Arya et al. 1993). The difficulty of regenerated ginseng plants in forming roots might be related to the structural abnormality of the somatic embryos, since morphologically abnormal somatic embryos such as multicotyledonary and multiple embryos also have been frequently observed (Butenko et al. 1968; Chang and Hsing 1980; Lee et al. 1990; Arya et al. 1991, 1993). We suggest that the difficult root formation of ginseng plantlets is the main hinderance for normal plant regeneration to plants viable under field conditions. Therefore, somatic single embryogenesis from mature cotyledon explants may be an efficient procedure for obtaining the normal plant regeneration with both shoot and root systems.

Even though the ginseng plants derived from single embryos were regenerated normally, balanced root and shoot growth was not easily achieved in ginseng plants regenerated from somatic single embryos on half-strength MS medium. In general, half-strength medium is used for plant

growth. When the ammonium nitrate in the MS medium was omitted, the root growth of the ginseng plants was enhanced. We suggest that ammonium nitrate is not appropriate for stimulating root growth in ginseng plants. It has been reported that high levels of ammonium nitrate highly suppressed adventitious root development but promoted somatic embryo production during the culture of carrot cell clumps (Halperin 1966).

When the cotyledon explants of *Panax ginseng* were cultured on growth regulator-free medium, multiple or single embryos were formed, depending on the degree of maturity of the zygotic embryos, and only the single embryos developed into morphologically normal plants. The above results indicate that a high frequency of somatic single embryos can be obtained easily by culturing cotyledon explants of mature zygotic embryos on growth regulator-free medium. In most *Panax ginseng* tissue cultures, somatic embryos were developed from callus on medium with exogenous growth regulators (Chang and Hsing 1980; Shoyama et al. 1988; Cellarova et al. 1992; Arya et al. 1993). However, in the culture of ginseng cotyledons on medium with exogenous growth regulators such as 2,4-D (2,4-dichlorophenoxy-acetic acid), callus was formed from the explants; thus in this medium, it may not easy to obtain the high frequency of direct somatic single embryogenesis. The direct single embryos formed on growth regulator-free medium were closely similar in morphology to the zygotic embryos of ginseng, and the regenerated plants from single embryos were successfully acclimatized in a greenhouse. A similar result was reported in carrot tissue cultures; somatic embryos from the apical meristem induced by osmotic stress regenerated more normally into plants than somatic embryos induced by 2,4-D treatment (Kamada et al. 1989). On the basis of the present experiment, we suggest that direct somatic single embryogenesis on growth regulator-free medium can be applied to an advanced technique for successful plant regeneration from Korean ginseng cotyledon cultures.

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