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Research article

In vitro grown thickened taproots, a new type of soil transplanting source in *Panax ginseng*

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ABSTRACT

Background: The low survival rate of *in vitro* regenerated *Panax ginseng* plantlets after transfer to soil is the main obstacle for their successful micropropagation and molecular breeding. In most cases, young plantlets converted from somatic embryos are transferred to soil.
Methods: *In vitro* thickened taproots, which were produced after prolonged culture of ginseng plantlets, were transferred to soil.
Results: Taproot thickening of plantlets occurred near hypocotyl and primary roots. Elevated concentration of sucrose in the medium stimulated the root thickening of plantlets. Senescence of shoots occurred following the prolonged culture of plantlets. Once the leaves of plantlets senesced, the buds on taproots developed a dormant tendency. Gibberellic acid treatment was required for dormancy breaking of the buds. Analysis of endogenous abscisic acid revealed that the content of abscisic acid in taproots with senescent shoots was comparatively higher than that of taproots with green shoots. Thickened taproots were transferred to soil, followed by exposure to gibberellic acid or a cold temperature of 2°C for 4 mo. Cold treatment of roots at 2°C for 4 mo resulted in bud sprouting in 84% of roots. Spraying of 100 mg/L gibberellic acid also induced the bud sprouting in 81% roots.
Conclusion: Soil transfer of dormant taproots of *P. ginseng* has advantages since they do not require an acclimatization procedure, humidity control of plants, and photoautotrophic growth, and a high soil survival rate was attained.

1. Introduction

Korean ginseng plants (*Panax ginseng* Meyer) are herbaceous medicinal plants that grow slowly. A cultivation period of > 4 yr is required before the plants set seeds. Therefore, plant tissue culture is an alternative method for the clonal propagation and breeding of ginseng. Plant regeneration via somatic embryogenesis in *P. ginseng* has been reported [1–6]. However, there are a few reports of successful field transfer of tissue culture-raised ginseng plantlets [3,7]. Serious problems are encountered during acclimatization and soil/field transfer of plantlets. The *in vitro* grown plantlets are feeble and can be affected by fungal infections [3,7].

P. ginseng is a perennial plant with storage taproots. The leaves and stems die at the end of the growing season. The storage taproots survive under the ground during winter, and new shoots develop from overwintering buds on taproots. Small ginseng taproots are mainly used as planting stocks for continuous cultivation in open fields.

We observed the *in vitro* taproot thickening of plantlets after prolonged culture. In this study, we investigated the effect of sucrose on the taproot thickening of *in vitro* grown plantlets, in addition to the effects of gibberellic acid (GA_3) and cold temperatures on the bud dormancy breaking of taproots. Soil transfer of *in vitro* grown thickened taproots of ginseng plantlets was successfully accomplished.

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2. Materials and methods

2.1. Germination and plant regeneration

Stratified seeds of Korean ginseng were harvested from a research field of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science (Chungcheongbuk-do, Korea), RDA. Embryogenic callus and somatic embryogenesis were established as described earlier [7]. Germination of somatic embryos was induced by transferring embryos onto 1/2 Schenk and Hildebrandt (SH) medium with 5 mg/L GA_3. Plantlets were maintained on 1/2 strength SH [8] medium with 3% sucrose for several months without subculture. During this prolonged culture period, leaf senescence and thickening of roots were monitored, and photographs were taken if necessary. To investigate the role of GA_3 on the bud growth on taproots, defoliated taproots were transferred onto 1/2 SH medium with different concentrations (0 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, and 40 mg/L) of GA_3. After 1 mo of culture, the growth of shoots and roots was assessed. Four plants were cultured in polycarbonate Magenta culture vessels (Sigma Chemical Co., St Louis, MO, USA). Twenty explants were used per treatment, and each treatment was repeated three times. Plantlets were cultured in polycarbonate Magenta culture box or 300 mL glass bottles.

2.2. Effect of sucrose on the growth of taproots

Somatic embryo-derived plantlets with roots and shoots (approximately 10 mm in length) were transferred onto 1/2 SH medium with various concentrations of sucrose (0%, 1%, 2%, 3%, 4%, and 5%) in a 500 mL recipient for automated temporary immersion (RITA) bioreactor (Cirad Biotrop, Montpellier Cedex, France). After 2 mo of culturing, the growth of roots and shoots was monitored by measuring the lengths of shoots and roots and the diameters of roots. Twenty explants were cultured in the bioreactor, and each treatment was repeated three times.

2.3. Soil transfer of thickened taproots after cold and GA_3 treatment

Taproots with dormant buds (3 mm diameter of roots) were transferred to soil in plastic square boxes ($25 \times 20 \times 15$ cm^3) containing perlite and peat (3:1 v/v), and then treated with 100 mg/L GA_3 or exposed to a cold temperature of 2°C or 10°C for 4 mo. The rate of sprouting was monitored after transfer of the soil boxes to a culture room maintained at 24°C. Fifteen roots were planted in a box. Thirty roots were used per treatment, and each treatment was repeated three times.

2.4. Abscisic acid analysis

Plantlets at various stages of development (cotyledonary somatic embryos, germinated embryos, taproots with green shoots, and taproots with senescent shoots) were sampled for the analysis of endogenous abscisic acid (ABA) content. Samples were ground in a mortar with liquid nitrogen, and the powdered samples were transferred into a tube and mixed with 80% methanol with 1% acetic acid and butylated hydroxytoluene. After vortexing of liquids for 1 min and sonication for 10 min at 0°C, the sample mixtures were centrifuged at 19,750 *g*, 4°C, for 10 min. The supernatants were collected, and the pellets were re-extracted with 3 mL of the extraction solvent. The second group of extracts was centrifuged, and then supernatants were combined and dried completely in a rotavapor.

The extracts were reconstituted in 200 μL of acetonitrile/water/acetic acid (90:10:0.05, v/v), stirred, vortexed, centrifuged (13,167 *g*, 10 min), and filtered through a 0.45 μm PTFE filter (Waters, Milford, MA, USA), and 10 μL of the extracts were injected into an liquid chromatography-ion trap-time of flight-mass spectrometer (LC-IT-TOF-MS) system (Shimadzu, Kyoto, Japan) equipped with an ESI source used in positive and negative ion modes. Quantification was performed using the standard addition method by spiking control plant samples with ABA solutions (ranging from 10 ng/mL to 200 ng/mL).

LC experiments were conducted on a Shimadzu (Kyoto, Japan) HPLC system that consisted of an LC-20AD binary pump, a DGU-20A degasser, an SIL-20A autosampler, a CTO-20AC column oven, and an SPD-M20A PDA detector. The mobile phase (delivered at 0.5 mL/min) comprised solvent A (H_2O) and solvent B (CH_3CN). A binary gradient elution was performed for ABA analysis: initial 25% B for 1.0 min, linear gradient 25–35% B from 2 min to 5 min, linear gradient 35–55% B from 6 min to 15 min, linear gradient 55–70% B from 16 min to 20 min, and a final quick return to the initial 25% B, which was maintained for 10 min to balance the column. Chromatographic separation was achieved on an Eclipse XDB-C18 column (5 μm, 3.0×250 mm^2; Agilent, Santa Clara, USA) at 40°C.

The LC-IT-TOF-MS mass spectrometer (Shimadzu) equipped with an ESI source was used in the negative ion mode. The following optimized analytical conditions were used: detector voltage, 1.60 kV; nebulizing gas (N_2) flow, 1.5 L/min; dry gas (N_2) flow, 50 kPa; pressure of TOF region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; and ion accumulation time, 30 ms. For the qualitative analysis, the scan ranges were set at *m/z* 100–500 for MS^1; ultra-high-purity argon was used for cooling. Standard ABA was directly examined under the same conditions.

3. Results and discussion

3.1. In vitro taproot thickening of plantlets converted from somatic embryos

Somatic embryos germinated rapidly and converted into plantlets on transfer to the medium supplemented with more than 5 mg/L GA_3 (Fig. 1A). Germinating somatic embryos were transferred onto 1/2 SH medium with 3% sucrose. Rapid growth of shoots and roots was achieved within 2 wk of culture (Figs. 1B, 1C). Following elongation of the plantlets, thickening of taproots was observed in the hypocotyl and primary root regions (Fig. 1D). The plantlets exhibited obviously thickened taproots with well-developed shoots (Figs. 1E, 1F). In plants with fleshy taproots, such as ginseng, carrot, radish, and beet, primary roots with hypocotyls become enlarged and function as storage taproot organs [9]. We found that *in vitro* cultured ginseng plantlets exhibited the same pattern of root thickening as that observed in taproot formation in the other plants.

3.2. Effect of sucrose on the growth of plantlets and root thickening

Small plantlets, just after germination of somatic embryos, were transferred into an RITA temporary immersion bioreactor (Fig. 2A) containing various concentrations (1%, 2%, 3%, 4%, and 5%) of sucrose. Fresh weights of shoots and roots were affected by the sucrose concentration. At a high sucrose concentration (5%), the fresh weight of roots was promoted but that of shoots (Figs. 2B–2D) was reduced. Accordingly, the growth of plantlets and root thickening were highly affected by the sugar concentration in the medium. As the sucrose concentration increased, the length of shoots and roots of plantlets were reduced (Fig. 3A), but root thickening was conspicuously enhanced (Fig. 3B). These results suggest that an excess amount of sucrose absorbed by plantlets may stimulate the secondary growth of roots. In *Lilium*,

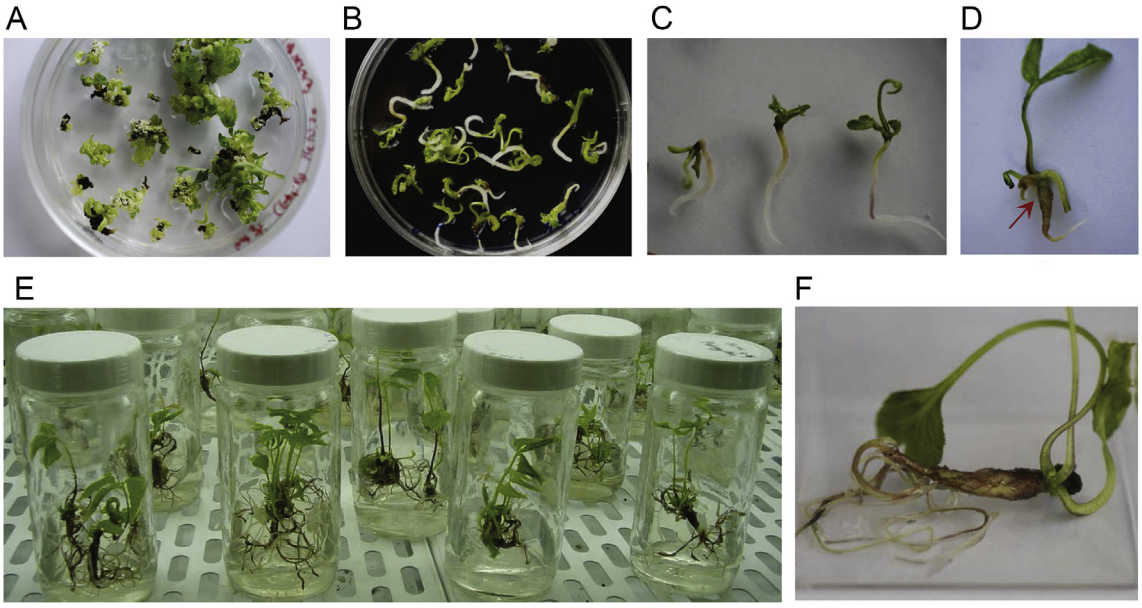

Fig. 1. Thickened taproot formation of plantlets regenerated from somatic embryos of *Panax ginseng*. (A) Germinating somatic embryos on medium with 5 mg/L GA_3. (B) Separation of germinating somatic embryos from embryo clusters. (C) Small plantlets after 1 mo of culture. (D) Thickening (arrow) of roots in the hypocotyl and primary root regions after 2 mo of culture. (E) Fully grown plantlets with thickened roots after 3 mo of culture. (F) An enlarged view of a plantlet with a thickened taproot with green leaves. GA_3, gibberellic acid.

a higher concentration of sucrose has been shown to increase the size of bulb scales. The combination of sucrose and mannose has also been demonstrated to promote bulb growth [10]. In shoot cultures of yam (*Dioscorea composita*), 8% sucrose was found to be the most significant medium constituent for promoting micro-tuber induction [11].

3.3. Dormant taproot formation after senescence of plantlets

Ginseng plants are perennial plants with storage taproots. Leaves of naturally grown ginseng plants age during the fall. In this study, a similar pattern of leaf senescence occurred in the *in vitro* cultured ginseng plantlets derived from somatic embryos. The

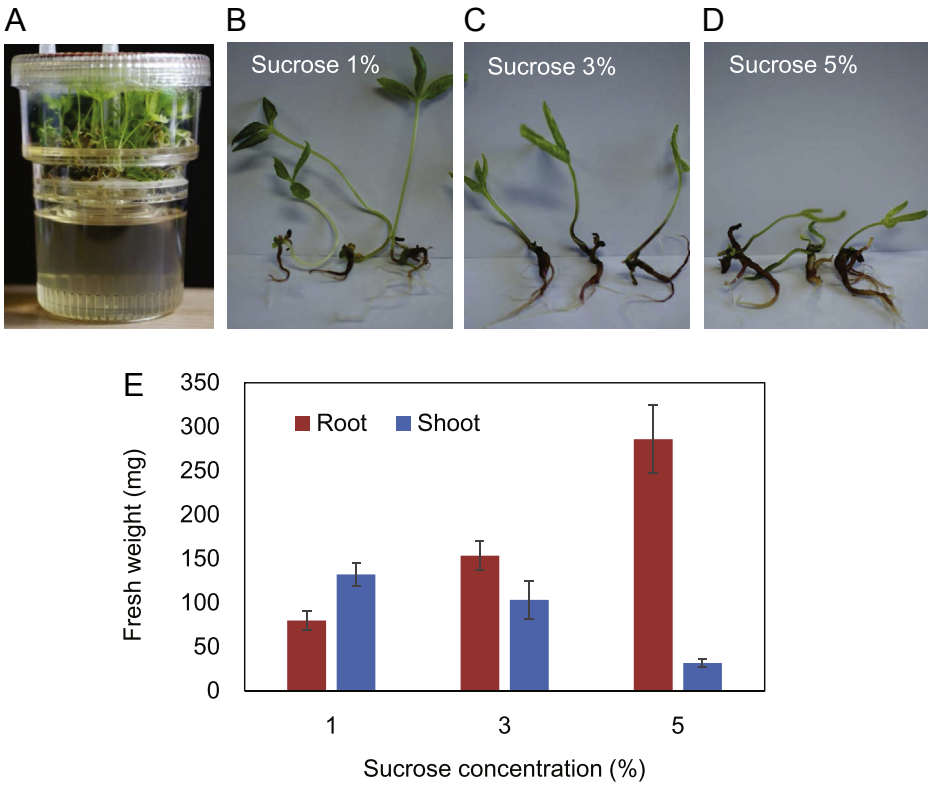

Fig. 2. Effects of sucrose concentration on the shoot and root weights of plantlets cultured in the RITA bioreactor. (A) Photograph of ginseng plantlets grown in the RITA bioreactor. (B–D) Photographs of plantlets grown in different sucrose concentrations in the RITA bioreactor after 2 mo of culture. (E) Fresh weights of roots and shoots of plantlets cultured in the RITA bioreactor in different sucrose concentrations. Vertical bars indicate the mean ± SE of three independent samples. RITA, recipient for automated temporary immersion; SE, standard error.

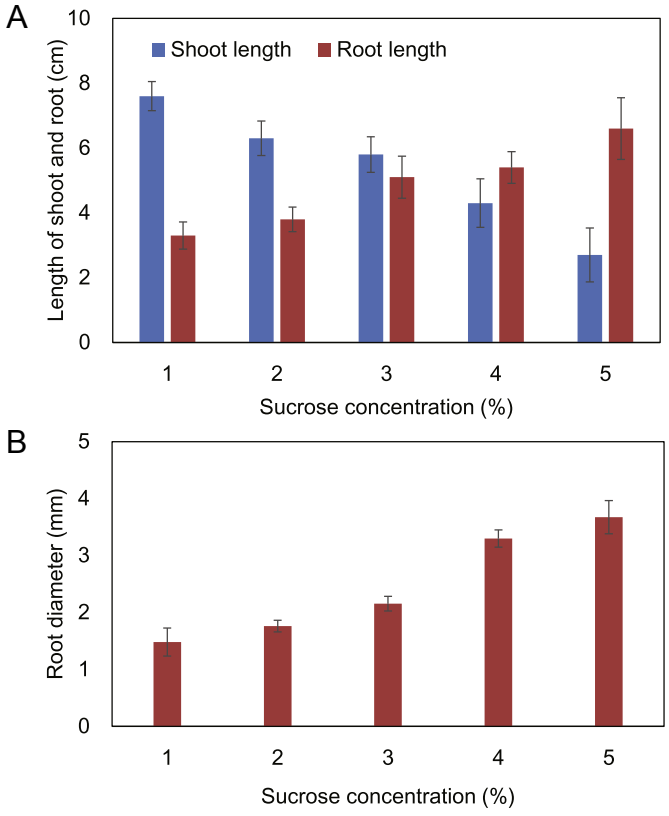

Fig. 3. Growth of plantlets in medium with different concentrations of sucrose (1%, 2%, 3%, 4%, and 5%) after 2 mo of culture. (A) Growth of shoots and roots in the RITA bioreactor. (B) Diameters of roots of plantlets cultured in various concentrations of sucrose. Vertical bars indicate the mean ± SE of three independent samples. RITA, recipient for automated temporary immersion; SE, standard error.

shoot parts (leaves) of plantlets turned yellow after prolonged culture (more than 3 mo), indicative of the senescence of leaves. Thickened taproots with tiny buds were obtained after leaf senescence (Fig. 4A). All the taproots had obvious shoot buds on their heads (Fig. 4B). Although the taproots with buds were subcultured on a new hormone-free medium, growth of shoots from buds did not occur (Fig. 4C). This result indicates that bud dormancy of taproots also occurred when the leaves died following prolonged culturing. GA_3 was a prerequisite for the bud sprouting of these taproots. The optimal GA_3 concentration for the sprouting of buds, which is a suitable concentration for the breaking of buds, was 10 mg/L (Figs. 4D, 4E). Owing to their dormancy, taproots can be preserved for > 6 mo. All the dormant taproots regenerated into plantlets after the GA_3 treatment.

Leaf senescence in plants grown in nature can occur prematurely under unfavorable environmental conditions [12]. The primary reason for leaf senescence in *P. ginseng* plantlets might be the deprivation of nutrition in the medium because, in this study, senescence occurred only after prolonged culture. Senescence and dormancy of plantlets caused by deficiency of nutrients have been reported in bulbous plant species when they are not subcultured [13].

Various phytohormones, including ABA, salicylic acid (SA), jasmonic acid (JA), and ethylene, are associated with leaf senescence. ABA is considered a plant stress hormone. It has been well known that ABA treatment stimulates leaf senescence [14]. Dormancy of plantlets occurs frequently during *in vitro* culture [15]. Leaf senescence and dormancy of buds on thickened ginseng roots might be attributed to endogenous ABA accumulation in plantlets after prolonged culture.

Endogenous ABA content was highest in taproots with senescent leaves (78.5 ng/g FW) compared with those with green shoots (34.2 ng/g FW) (Fig. 5). ABA content in taproots with senescent leaves was remarkably reduced (38.4 ng/g FW) after 4 mo of cold treatment at 2°C (Fig. 5). Germinating somatic embryos induced by GA_3 treatment had the lowest content of ABA (12.7 ng/g FW). This result revealed that the dormancy of buds of taproots with senescent leaves might be attributed to the relatively high content of endogenous ABA.

3.4. Soil transfer of in vitro thickened taproots

After the removal of aged leaves, all thickened taproots with healthy dormant buds (Fig. 6A) were transferred to soil, and were then exposed to a cold temperature of 2°C or 10°C for different

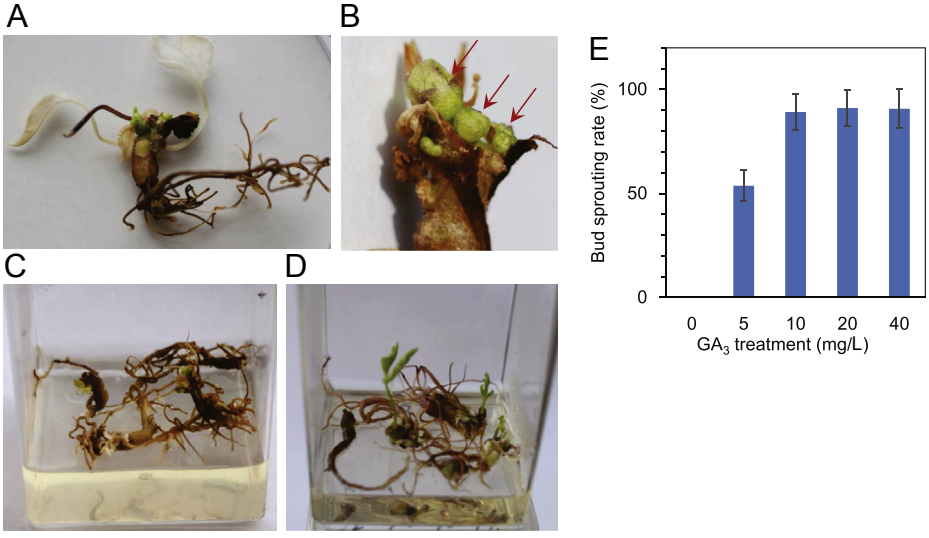

Fig. 4. Bud dormancy and sprouting of thickened taproots with senescent leaves. (A) Thickened taproots with senescent leaves after prolonged culturing (for 3 mo) of plantlets. (B) An enlarged view of the head portions of roots with dormant buds (arrows). (C) No sprouting of buds occurred, even after transfer onto a fresh medium. (D) Sprouting of buds after treatment with 10 mg/L GA_3. Vertical bars indicate the mean ± SE of three independent samples. (E) GA_3 treatment against bud sprouting rates. GA_3, gibberellic acid; SE, standard error.

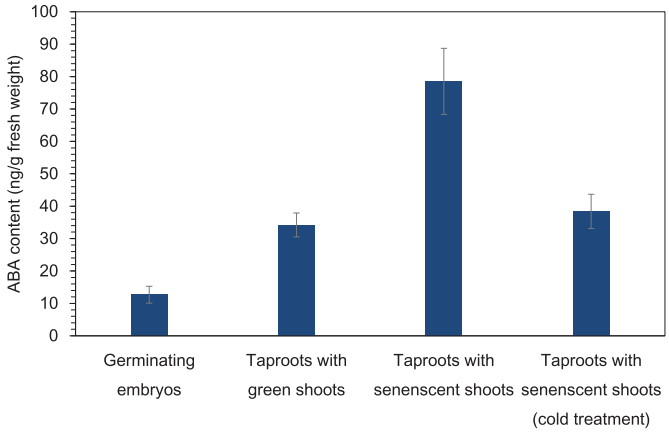

Fig. 5. Analysis of endogenous ABA contents in various developing stages of ginseng plantlets. Endogenous ABA contents in germinating embryos, taproots with green shoots, and taproots with senescent shoots, and taproots with senescent shoots after 4 mo of cold treatment at 2°C. Vertical bars indicate the mean ± SE of three independent samples. ABA, abscisic acid; FW, fresh weight; SE, standard error.

durations or treated with GA_3. The taproots did not produce any shoots unless they were exposed to either GA_3 or a cold temperature (Fig. 6B). When pots containing taproots were exposed to a cold temperature (2°C) for 4 mo and then returned to room temperature, bud sprouting occurred on 84% of taproots (Figs. 6C, 6D, 6F). Cold treatment at 10°C was not effective for the dormancy breaking of buds because only 37% of roots exhibited sprouting after 4 mo of this treatment. Spraying of water containing 100 mg/L GA_3 also effectively induced the sprouting of buds on 81% of taproots (Fig. 6E). Spraying of GA_3 at a concentration of 20 mg/L or 40 mg/L was not effective because the sprouting of buds did not exceed 50% (data not shown). There are some differences in the number of sprouting leaflets between the cold and GA_3 treatment. Only a single leaflet developed on the taproots after cold treatment (Figs. 6C, 6D), but two leaflets were common after GA_3 treatment (Fig. 6E).

Choi et al. [3] described the transfer of actively growing *in vitro* ginseng plantlets to soil. Only 27% of the plantlets survived for up to 15 wk after transfer to a greenhouse [3]. The soil survival rate achieved by the soil transfer of *in vitro* thickened taproots was markedly increased compared with that observed with the transfer of young plantlets [3]. Dormant taproots can be preserved for a long period of time *in vitro*, and they can very easily be transferred to soil.

During *in vitro* culture, plantlets grow under very special conditions in relatively airtight cultivation vessels (high humidity) and a carbon-rich condition, mainly in the sucrose medium as an energy source. After *ex vitro* transfer, plantlets might easily be impaired by sudden changes under low humidity and photoautotrophic condition, therefore transplantation of plantlets usually needs some period of acclimatization with gradual lowering in air humidity [16]. In the soil transfer of thickened taproots of ginseng, special treatment for humidity control is not required for *ex vitro* acclimatization because shoot sprouting occurs after *ex vitro* transfer. Thus, the soil transfer of *in vitro* thickened taproots may have several advantages for increasing the soil survival of *in vitro* propagated *P. ginseng* plantlets. There are 12 recognized ginseng species in the world depending on the method of classification [17]. This protocol can also be adopted for soil transfer of micropropagated plants of other ginseng species.

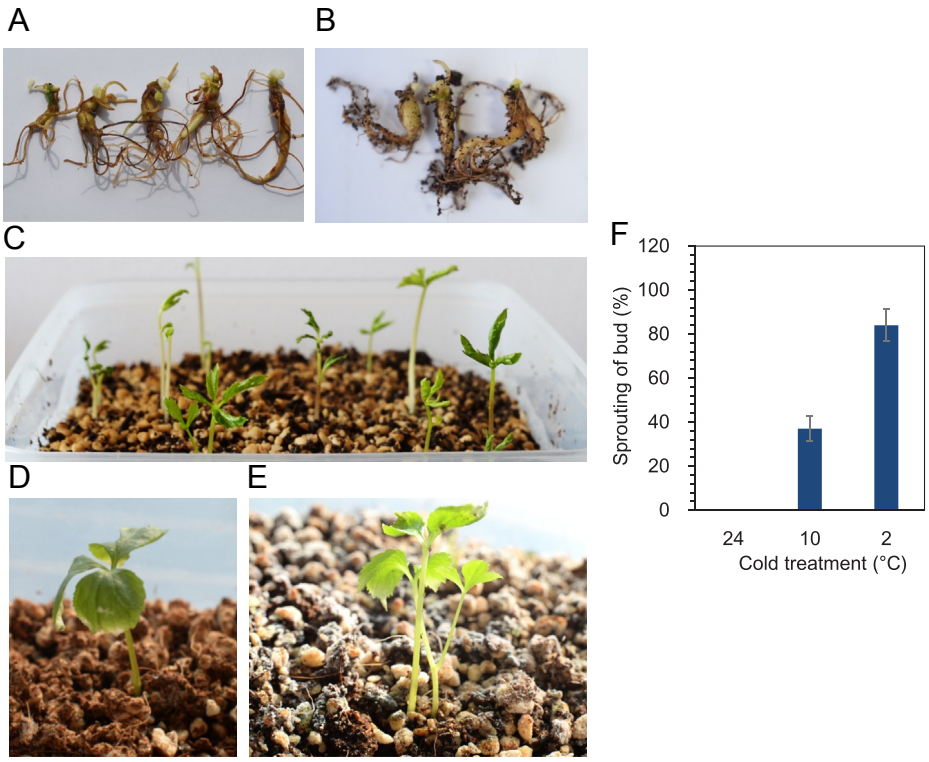

Fig. 6. Soil transfer of thickened taproots after cold or GA_3 treatment. (A) Tuberous roots with dormant shoot buds before soil transfer. (B) No sprouting of taproots without cold and GA_3 treatment after 4 mo of soil transfer. (C, D) Sprouting of a bud on a taproot after cold treatment at 2°C for 4 mo. (E) Sprouting of two leaves on a taproot after spraying with 100 mg/L GA_3. (F) Sprouting rates of soil-transferred taproots after exposure to different cold temperatures for 4 mo. Vertical bars indicate the mean ± SE of three independent samples. GA_3, gibberellic acid; SE, standard error.

Conflicts of interest

The authors declare that they have no conflicts of interest and that the presented work is compliant with the ethical standards of *Journal of Ginseng Research*.

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References

[1] Chang WC, Hsing YI. Plant regeneration through somatic embryogenesis in root-derived callus of ginseng (*Panax ginseng* C.A. Meyer). Theor Appl Genet 1980;57:133–5.
[2] Ahn IO, Le BV, Gendy C, Tran Thanh Van K. Direct somatic embryogenesis through thin cell layer culture of *Panax ginseng*. Plant Cell Tissue Organ Cult 1996;45:237–43.
[3] Choi YE, Yang DC, Park JC, Soh WY, Choi KT. Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormone-free medium. Plant Cell Rep 1998;17:544–51.
[4] Tang W. High-frequency plant regeneration via somatic embryogenesis and organogenesis and *in vitro* flowering of regenerated plantlets in *Panax ginseng*. Plant Cell Rep 2000;19:727–32.
[5] Teng WL, Sin T, Teng MC. Explant preparation affects culture initiation and regeneration of *Panax ginseng* and *Panax quinquefolius*. Plant Cell Tiss Org Cult 2002;68:233–9.
[6] Kim YJ, Lee OR, Kim KT, Yang DC. High frequency of plant regeneration through cyclic secondary somatic embryogenesis in *Panax ginseng*. J Ginseng Res 2012;36:442–8.
[7] Choi YE, Jeong JH, In JK, Yang DC. Production of herbicide-resistant transgenic *Panax ginseng* through the introduction of phosphinotricin acetyl transferase gene and successful soil transfer. Plant Cell Rep 2003;21:563–8.
[8] Schenk RU, Hildebrandt AC. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 1972;50:199–204.
[9] Kawakami K. Physiology of yield of underground storage organs. In: Gupta US, editor. Crop physiology. New Delhi: Oxford and IBH Publishing Co.; 1978. p. 269–309.
[10] Yamagishi M. Effects of mannose on enlargement of *in vitro* bulblets of *Lilium japonicum* Thunb. Bull RIAR Agr Coll 1995;4:86–9.
[11] Alizadeh S, Mantell SH, Viana AM. *In vitro* shoot culture and microtuber induction in the steroid yam *Dioscorea composita* Hemsl. Plant Cell Tiss Org Cult 1998;53:107–12.
[12] Lim PO, Woo HR, Nam HG. Molecular genetics of leaf senescence in *Arabidopsis*. Trends Plant Sci 2003;8:272–8.
[13] Hussey G, Falavigna A. Origin and production of *in vitro* adventitious shoots in the onion, *Allium cepa* L. J Exp Bot 1980;31:1675–86.
[14] Zeevaart JAD, Creelman RA. Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol 1988;39:439–73.
[15] Delvallée I, Paffen A, De Klerk GJ. The development of dormancy in bulblets of *Lilium speciosum* generated *in vitro*. II. The effect of temperature. Physiol Plant 1990;80:431–6.
[16] Chandra S, Bandopadhyay R, Kumar V, Chandra R. Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol Lett 2010;32:1199–205.
[17] Wen J, Zimmer EA. Phylogeny and biogeography of *Panax* L. (the ginseng genus, Araliaceae): inferences from ITS sequences of nuclear ribosomal DNA. Mol Phylogenet Evol 1996;6:167–77.