ORIGINAL ARTICLE

Establishment of a qualified ex vitro-acclimatized whole plant reproduction system using secondary somatic embryos in *Panax ginseng*

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Abstract
In vitro regenerated ginseng plants from embryos have been successfully ex vitro acclimatized and transplanted to soil as reported in previous studies. However, resprouting of ex vitro acclimatized ginseng in the following year has not been achieved. In this study, we optimized plant regeneration protocol through the somatic embryo culture and acclimatization steps in the soil for Korean ginseng. Primary somatic embryos were induced from the cut cotyledons in hormone-free MS medium with 3% sucrose after 8 weeks. The secondary somatic embryos were induced from transplanted primary embryos in hormone-supplemented MS containing 1 mg/L 2,4-D and 0.5 mg/L BAP for 10 weeks. Two weeks more cultivation in the same medium generated shoots, and shoot elongation and the formation of all above-ground parts of the shoots was achieved in hormone-free MS medium over 6 weeks. Roots were formed in 1/2 MS medium with 1% sucrose and 0.3% of activated charcoal after 12 weeks. Overall, plant generation, from the cotyledon of the zygote to the transplantable plantlet, was about 53% efficient (33 plantlet/61 secondary embryos) and took about 38 weeks. The acclimatization of transplanted plantlets in a soil mixture was 82% efficient. Buds on taproots of adapted plants developed a dormant tendency, and the dormancy of these buds was broken during cold treatment at 4 °C for 3 to 4 months. The system we developed can be effectively utilized for the mass production of Korean ginseng through somatic embryo culture.

Keywords *Panax ginseng* · Somatic embryo · Secondary embryo · Ex vitro acclimatization

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Introduction

Korean ginseng (*Panax ginseng* Meyer) is a perennial herbaceous plant belonging to the genus *Panax* of the Araliaceae family (Kim et al. 2014). It has a long history of medicinal use and is attracting attention as a high-value-added crop worldwide (Brekhman 1957; Qi et al. 2011). The pharmacological efficacy of saponin, a representative secondary metabolite, in particular has been proven (Brekhman 1957; Qi et al. 2011). For a crop, ginseng has a very slow growth rate and a very long juvenile phase; It normally takes 3 to 4 years to obtain seeds (Zhang et al. 2014), of which only 40 to 60 seeds are produced from one mature individual inflorescence. The ginseng embryos in the harvested seeds are immature, having reached only the globular stage, and are less than 200 μm in any aspect (Ahn 1996; Choi et al. 1998a, 1998b). In addition, due to the immature embryo and the dormancy characteristic of the ginseng seeds, both artificial seed scarification and stratification that have been just called a stratification are required at 15~20 °C (Lee et al. 2014). After the stratification,

an additional 100 days of cold treatment at 0 ~ 4 °C is further needed to break seed dormancy. These physiological conditions are a major hindrance to ginseng cultivation and production. They also affect the breeding efficiency since it takes a long time to fix one generation (Lee et al. 1986; Won et al. 1988; Kwon et al. 2001; Bang et al. 2020). Generally, after a period of stratification and dormancy breaking, seeds whose embryos are still in the globular stage have been used for soil and in vitro germination. It is known that prolonged this period, which is performed primarily for in vitro experimental purposes, results in lower germination. However, there are no reports yet testing in vitro fully-matured cotyledonary stage of ginseng embryos after stratification and breaking dormancy. Thus, the establishment of robust and stable in vitro tissue culture and ex vitro acclimatization systems using fully matured embryos for ginseng production is required.

Plant somatic embryos are derived from somatic cells that can develop into young plants through a series of morphological changes, a process very similar to that of zygote embryos (Dodeman et al. 1997). Secondary somatic embryos are derived from the previously formed primary embryos, have a high proliferation rate, and can be kept viable by repeated culturing thus having the advantage of long-term maintainability (Raemakers et al. 1995). Therefore, in vitro propagation producing large quantities of plants through the generation of secondary somatic embryos can shortcut the long production life cycles. The in vitro propagation is also more applicable in biotechnologies. It has been reported that ginseng somatic embryos are relatively easy to form; primary embryos have been formed mainly from cotyledon slices derived from zygote embryos, and cell differentiation has been achieved (Choi et al. 1998a, 1998b, 1999, 2001; Choi and Soh 1997; Tang 2000; Kim et al. 2012). There are many reports that primary embryos formed from ginseng can be used to form secondary embryos, and cell differentiation was achieved in larger quantities at this stage as well (Choi et al. 2003; Zhou and Brown 2006; Lee et al. 2008; Kim et al. 2016). However, there have been no reports of cases in which in vitro-regenerated ginseng plants were successfully transplanted into soil, cultivated in the field, and progressed to the next generation showing the perennial characteristic of root sprouting. In this study, we report acclimatization conditions that generate plantlets capable of producing viable root-bud embryos, and thus capable of resprouting after the first growing season, by optimizing the in vitro conditions for somatic embryo production and regeneration starting from fully matured seed embryos.

Materials and methods

Plant materials

Korean ginseng seeds (*Panax ginseng* Meyer, Yunpoong cultivar) were obtained from the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science (Chungcheongbuk-do, Korea), the Rural Development Administration (RDA), and kept in moist storage at ± 1 °C. Seeds with a 3 mm root (in the fully mature cotyledonary stage of the embryo which is 6 ~ 8 mm in total length) were selected, and seed coats were removed. The naked seeds were surface-sterilized in 70% ethanol for 1 min, followed by a 50% solution of commercial bleach for 20 min with vigorous hand-shaking for 5 min and a shaker at 260 rpm for 15 min, then washed four times with sterilized distilled water.

In vitro induction of primary somatic embryos

Cotyledons of mature zygotic embryos were excised from the seeds and cultured on an induction medium. This consisted of MS medium (Murashige and Skoog. 1962) including vitamins, 3% sucrose, and 0.8% agar with or without synthetic hormones: 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (BAP). The pH of all media was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Cotyledon explants were each placed into a plant culture dish (model 310,100; SPL Life Sciences, Korea;100 mm × 40 mm) containing 50 ml of medium. Fifteen explants were cultured per dish and each treatment was replicated 3 times (n = 45). All cultures were maintained under dark conditions at 25 ± 2°C for 8 weeks.

Induction of secondary somatic embryos

Embryos grown to 4 mm in length or longer were isolated from the primary somatic embryos generated on the hormone-free medium and cultured in one of the same two mediums used for the primary embryo induction under low-light conditions (50 µmol m^{-2} s^{-1}) using fluorescent lamps for 2 weeks. To induce secondary somatic embryos, 10–12 primary embryos were cultured per petri dish. A total of 41 primary somatic embryos were cultured in hormone-free medium and 43 primary somatic embryos were cultured in hormone-supplemented medium containing 2,4-D and BAP. Following low-light incubation, the embryos were cultured for 10 weeks under the same conditions adopted for primary embryo induction before starting the plant regeneration process.

Plant regeneration from secondary somatic embryos

The secondary embryos cultured on the hormone-supplemented medium were incubated under low-light conditions (50 µmol m^{-2} s^{-1}) using fluorescent lamps for 2 weeks. For

shoot induction, the secondary embryos (5 mm long) were separated from the primary embryos and cultured on the same medium. Growing embryos with shoots 10 mm long or more were transferred onto a hormone-free MS medium with 4% sucrose and 0.8% plant agar for shoot elongation. Shoots of embryos reaching about 3 cm or more were transferred to a tissue culture vessel (model 310,071; SPL Life Sciences, Korea) (72 mm × 72 mm × 100 mm) containing 70 ml of hormone-free 1/2 MS medium supplemented with 1% sucrose, 0.3% activated charcoal (Charcoal activated powder, JUNSEI) and 0.8% plant agar and cultured for 10 ~ 12 weeks to induce roots and promote plant development. During these last two culturing steps, the light intensity was slowly increased to 80 μmol m^{-2} s^{-1}.

Acclimatization of in vitro regenerated plants

The roots of well-developed in vitro ginseng plants were gently washed of medium and transplanted into plastic pots containing soil mixed with peat moss (Lettisher Wei Btorf, Letta-Flor) and perlite (SJ Korea) (5:1 v/v). The plants were covered with a transparent lid to provide a high relative humidity in the pots for about 7 days, and the lid was then gradually opened over 1 ~ 2 weeks to allow plants to acclimatize to natural environmental conditions (ex vitro).

Cultivation of regenerated plants and low-temperature storage of their roots

Following the acclimatization process, surviving ginseng plants in the soil mixed with peat moss and pearlite (5:1; v/v), grew for more than 2 months before the onset of leaf senescence. Shoot parts were then removed and planted in the watered soil mixture. Then, the plantlets were exposed to a cold temperature of 4℃ for about 4 months. The rate of dormant bud formation and sprouting from dormant buds on the tap roots was investigated after the cold treatment.

Results and discussion

Induction of primary somatic embryos from excised cotyledons of mature zygotic embryos

Ginseng is a typical immature plant. After harvest, it is subjected to a stratification process for four months at 15℃, followed by cold treatment for 100 days at 2 ~ 4℃ to break dormancy and start germination from February the following year (Lee et al. 2014). The embryos reached a length of 6 ~ 8 mm with roots breaking through the seed coat and beginning to grow before they were used for further study (Fig. 1a and b, Fig. S1). It has been reported that embryos about 6 mm in length are at the fully matured cotyledonary stage (Choi et al. 1998a, 1998b). Thus, here we tested the viability of fully matured embryos (Fig. 1, Fig. S1c) for the somatic embryo development.

Plant tissue cultures were performed for about 2 months in hormone-free and hormone-supplemented MS medium (containing 1 mg/L 2,4-D and 0.5 mg/L BAP). In the hormone-free medium, most embryos were formed directly from the explants without callus formation (Fig. 1c and e). In the hormone-supplemented medium, embryos were formed by indirect somatic embryogenesis, in which the callus was formed first, and the embryo was formed from the callus (Fig. 1d and f). In the hormone-supplemented medium, the embryogenesis rate was 91% (n = 45) and an average of 15.3 embryos were formed per explant. In the hormone-free medium, the embryogenesis rate and the number of embryos per explant were 82% (n = 45) and 3.8 embryos, respectively. However, most of the embryos generated in the hormone-supplemented medium occurred as multi-embryos (Fig. 1d), and shoots were unevenly generated, which made them unsuitable for secondary embryo induction. This is similar to previous cases where the generation of somatic embryos increased in the hormone-supplemented medium, but they were not suitable for secondary embryogenesis (Choi et al. 1998b; Kim et al. 2012). On the other hand, embryos formed in the hormone-free medium had many single embryos (Fig. 1c), but after separating individual embryos, if cultured again in the hormone-free medium, shoots do not emerge and only the cotyledon expands. Thus, these tissues could be used to uniformly induce secondary embryos repeatedly.

Induction of secondary embryos from primary somatic embryos

In order to induce secondary embryos, cotyledon embryos that grew to more than 4 mm were isolated (Fig. 1e) from primary somatic embryos formed in the hormone-free medium and cultured for an additional 10 weeks more in both media. For those cultured in the hormone-free medium, the secondary embryo formation rate was 93.2% (n = 41), and the average number of secondary embryos per explant was 16.2 (total 619 secondary embryos: 41 × 93.2% × 16.2). In the hormone-supplemented medium, the secondary embryonic formation rate was 93.2% (n = 43) and the mean number of secondary embryos per explant was 11.2 (total 449 secondary embryos: 43 × 93.2% × 11.2). This shows that, in contrast to what was seen in the primary embryos, the number of secondary embryos per explant was decreased in the hormone-supplemented medium. Secondary embryos were generated on the surface of primary cotyledonary embryos, and the size of secondary embryos formed increased over the incubation period (Fig. 2a and b). The secondary embryos previously formed in the hormone-supplemented medium were relatively larger than those of
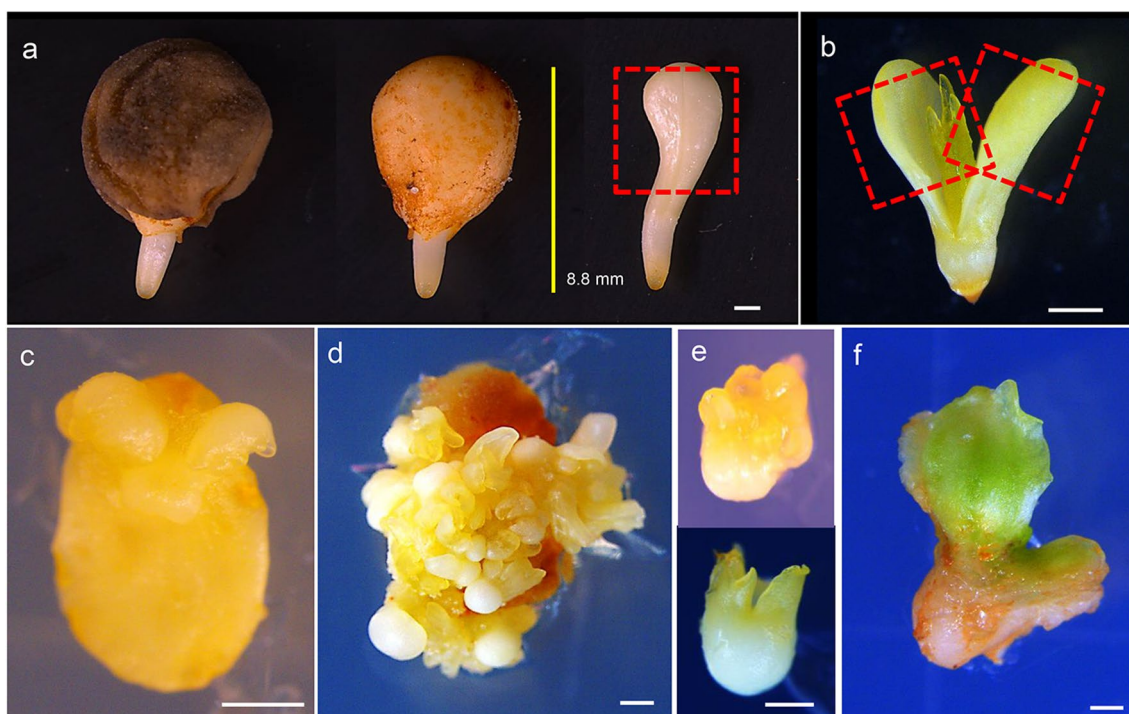
Fig. 1 Primary somatic embryo induction from the cotyledons of zygotic embryos. **a** The zygotic embryos dissected from stratified Korean ginseng seeds. **b** Front view of a zygotic embryo, from which cotyledons were excised and cultured. **c** Somatic primary embryogenesis from the cotyledon of zygotic embryos cultured on MS medium + 3% sucrose without hormones or **d** with 1 mg/L 2,4-D and 0.5 mg/L BAP after 8 weeks of culture. **e** Primary embryos isolated from hormone-free MS medium or hormone-supplemented MS medium. **f** Primary embryos cultured on hormone-free medium under low-light conditions for 1 week. Scale bars = 1 mm. The upper part of the embryo highlighted in the red box was used for somatic primary embryo induction

the hormone-free at the time of isolation, but by the end of the 10-week culture period, all secondary embryos grew to a length of about 3 mm regardless of origin (Fig. 2c). As described above, if embryos formed in the hormone-free medium are continuously cultured in the same medium, only the cotyledons become enlarged and shoots rarely emerge from the embryos. Therefore, this type of embryo is appropriate for maintaining the embryos at this stage as they can be excised at any time and transplanted to fresh hormone-free medium where the formation of new secondary embryos will occur. However, though the secondary embryo formation rate was slightly lower in the hormone-supplemented medium, the color of the embryos changed to green and the size of the embryos increased evenly after the second 2-week culturing in low light conditions as the light intensity increased from condition to 50 µmol m^{-2} s^{-1}. (Fig. 2d and e). This made these embryos grown on hormone-supplemented medium more suitable for plant regeneration.

Plant regeneration through induction of secondary embryo germination

For shoot elongation, 90 secondary embryos among 449 secondary embryos that had grown to more than 5 mm in size were isolated (Fig. 2e) and cultured in the same, hormone-supplemented medium. After 2 weeks, the embryo's volume increased and cotyledons developed (Fig. 3a). Shoots then began appearing between the cotyledons and developing (Fig. 3b). When they were cultured 4 weeks more, the shoot length had increased to about 1 cm (Fig. 3c). The rate of successful shooting was about 80% (Table 1). Individuals with these newly developed shoots were transferred to a hormone-free medium with 4% sucrose and further cultured for about four weeks. As the incubation proceeded, the shoot continued to elongate (Fig. 3d), and by the end of the incubation period, it grew to roughly 3 cm or more. By this point, the basal tuber area enlarged to a spherical shape with a diameter of about 5 to 10 mm (Fig. 3e). About 75% of individuals who started to develop shoots successfully grew to this stage (Table 1). In American ginseng which is closely related to Korean ginseng, higher concentrations of NH$_4^+$ inhibited the root development of ginseng (Zhou and Brown 2006), and the uptake of NH$_4^+$ is reduced when plants are cultured in medium containing activated charcoal (Eymar et al. 2000). Similarly, activated charcoal also has been used to induce the differentiation of somatic embryos and the development

Fig. 2 Secondary somatic embryogenesis from primary cotyledonary embryos. **a** A secondary embryo initiating on the surface of a primary cotyledonary embryo. **b** A secondary embryo after 2 weeks of culture. **c** Secondary embryos formed on a primary embryo cultured on MS medium with 3% sucrose, 1 mg/L 2,4-D, and 0.5 mg/L BAP for 10 weeks. **d** Secondary embryos growing under low light (50 µmol·m^{-2}·s.$^{-1}$) on the same medium. **e** Excised green secondary embryos separated after 2 weeks of culture under low-light conditions. Scale bars = 2 mm (**a**, **b**, and **e**), 1 cm (**c**, and **d**)
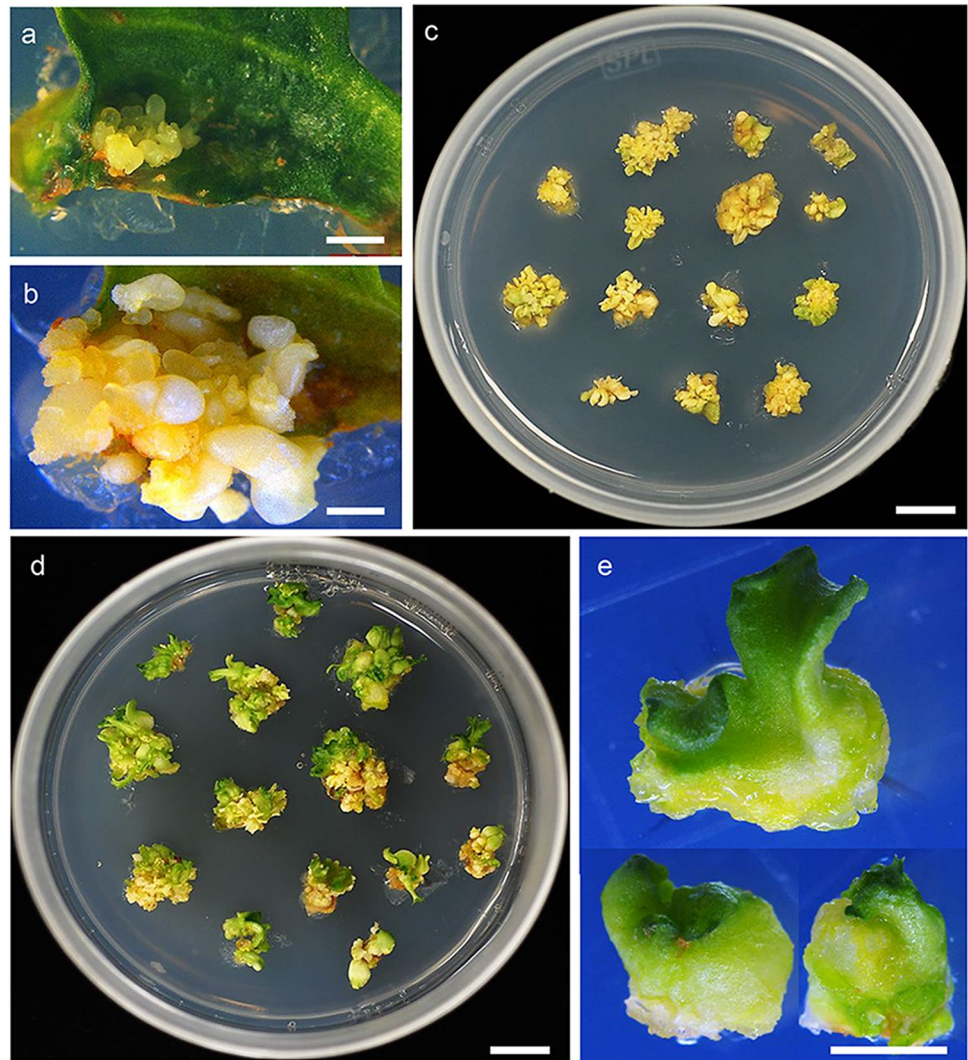
of plants by adding it to the medium (Zhou and Brown 2006; Lee et al. 2008; Kim et al. 2012). So, individuals with sufficiently elongated stems were transferred to hormone-free 1/2 MS medium supplemented with 1% sucrose and 0.3% activated charcoal and incubated for 8 weeks. During this time, the length of the shoots further extended to about 7 cm, and simultaneously, roots were induced from the basal tuber and the main root enlarged (Fig. 3f). Healthy fine roots developed from the fully thickened taproot, and dormant buds also developed top of the taproot (Fig. 3f). About 92% of the individuals transplanted to charcoal-containing media successfully developed through this stage (Fig. 3f, Table 1). These were transplanted to a soil mixture to begin the acclimatization process, preparing them ultimately for ex vitro acclimatization.

Establishment of soil acclimatization and germination of dormant embryos

Individuals that had developed as intact plantlets were washed with water and transplanted into plastic pots containing an unsterilized peat moss and perlite mix. For the first 7 days after transplantation, the plants were covered with a transparent lid, which was then opened little by little over the next 2 weeks to acclimatize the plants to ex vitro humidity levels (Fig. 4a). The aerial parts began to age after 3 to 6 months, and 82% of the plants produced dormant buds on the taproots (Fig. 4b, Table 1). During the following 4-month of low-temperature treatment at 4 °C, the size of the root increased, additional dormant buds were formed, and in some cases, dormant sprouting was identified (Fig. 4c and d). Buds that had sufficiently broken dormancy began to

Fig. 3 Whole plant regeneration from in vitro somatic embryos. **a** Isolated cotyledonary embryos cultured on MS medium with 3% sucrose, 1 mg/L 2,4-D, and 0.5 mg/L BAP under a light intensity of 40~50 μmol·m^{-2}·s^{-1}. **b** Green cotyledonary embryos shooting on the hormone-supplemented MS medium after 2 weeks of culture. **c** Plant regeneration step 1: Shooting on the hormone-supplemented MS medium after 4 weeks of culture. **d** Shoots elongating on hormone-free MS medium with 4% sucrose under a light intensity of 60~70 μmol·m^{-2}·s^{-1}. **e** Plant regeneration step 2: Elongated shoots with fully expanded leaves on the hormone-free MS medium after 4 weeks of culture. **f** Plant regeneration step 3: Rooted ginseng plants on ½ MS with 1% sucrose and 0.3% activated charcoal under a light intensity of 80 μmol·m^{-2}·s^{-1} after 8 weeks of culture. Scale bars = 10 mm

germinate as dormant embryos developed, and their stems grew out of the soil (Fig. S2a and b). After removing the soil, it was confirmed that the roots remained healthy and the above-ground part was also continuing to develop well (Fig. S2c). This result shows a typical case of an ex vitro-grown individual (Fig. S3): the above-ground parts began to senescence after about 6 months, and the dormant sprouts developed during the low-temperature treatment that break dormancy. According to previous reports, dormant roots were germinated by cold treatment of roots after in vitro acclimatization using sterilized soil (Lee et al. 2008; Kim et al. 2016, 2019). For ginseng root cultivation in greenhouse, peatmoss or cocopeat is mixed with perlite to adjust the physical properties and used for crop cultivation (Fig. 5).

The most commonly used mixed soil for domestic ginseng nursery is 70% peatmoss and 30% perlite (v/v) (Park et al. 2020). The soil used in this study is a mixture of 83% peat moss and 17% perlite, so it can be applied to field cultivation.

Efficiency of whole plant production through somatic embryogenesis in *Panax ginseng*

Looking at plant production in terms of efficiency, from one cotyledon, approximately 6 [2 (cotyledons) × 82% (percentage of cotyledons with embryos) × 4 (embryos per cotyledon)] embryos can be produced from two cotyledons (one seed) in 8 weeks and about 61 [6 (embryos) × 93% (percentage of explants with embryos) × 11 (embryos per explant)]

Table 1 The percent success for all three steps of the in vitro production of *Panax ginseng* Mayer plantlets and the final acclimatization process: frequencies of shooting (step 1), shoot elongation (step 2), taproot thickening (step 3), and ex vitro plant acclimatization

Efficiency (%)[1]			
Embryos Produced shoots (Step 1)	Shoot elongated (Step 2)	Plants with thickened taproot (Step 3)	Plants acclimatized in soil (dormant bud formation in soil)
80.2±6.2	75.0±4.1	91.5±6.1	81.9±5.2

[1]After shooting (Step 1), the "shoot elongated" percentage represents the frequency of shoots elongated (Step 2). After shoots elongated, the percentage of "plants with thickened taproot" represents the frequency of regenerated plants that exhibited taproot thickening (Step 3). The percentage of "plants acclimatized in soil" represents the frequency that plants with a thickened taproot survived in soil and formed dormant bud(s) on their taproots. For the analysis of percent success, total 90 (30×3 times) secondary embryos were used

embryos can be produced through secondary embryo production in another 12 weeks period. Finally, about 33 [61 (embryos)×80% (shoot induction rate)×75% (shoot elongation rate)×91% (thickened taproot induction rate)] plants that have thickened taproot from one stratified seed can be produced in about 38 weeks. After additional 24 weeks later, 26 plants [33 (plant with thickened taproot)×81% (acclimatization rate)] can be successfully completed acclimatization, enable to field cultivation.

Conclusion

In this study, we made intact plants surviving in ex vitro grown from somatic embryos based on previously reported optimal conditions. The purpose of this study was to increase the utilization of ginseng seeds after post-stratification and prolonged cold treatment. Although there are many cases of plants derived from somatic embryogenesis surviving through ex vitro acclimatization (Kim et al. 2012, 2016; Zhou and Brown. 2006), cyclic production of more than one generation has not been achieved in healthy state of soil-acclimatized roots. Thus, we show a method of cycled-induction generating plantlets capable of producing enlarged healthy main roots and the formation of dormant buds on the fully thickened taproot which kept their vitality after ex vitro acclimatization, a simulated growing season, and even produced sprouts after simulated winter conditions. The in vitro regenerated plantlets showed a high acclimatization rate of 82% when

Fig. 4 *Ex vitro* acclimatization of in vitro plants and dormant bud formation on taproots. **a** Plants with well-developed taproots transferred to a peat moss and perlite mixture (non-sterilized) for ex vitro acclimatization. **b** Taproots with dormant buds in the soil mixture after acclimatization. **c** Tuberous roots thickening with more adventitious buds during cold storage at 4℃ for 2 months. **d** Enlarged view of the tuberous roots in panel C. Scale bars = 2 cm (**a**), 1 cm (**b**, **c**, and **d**)
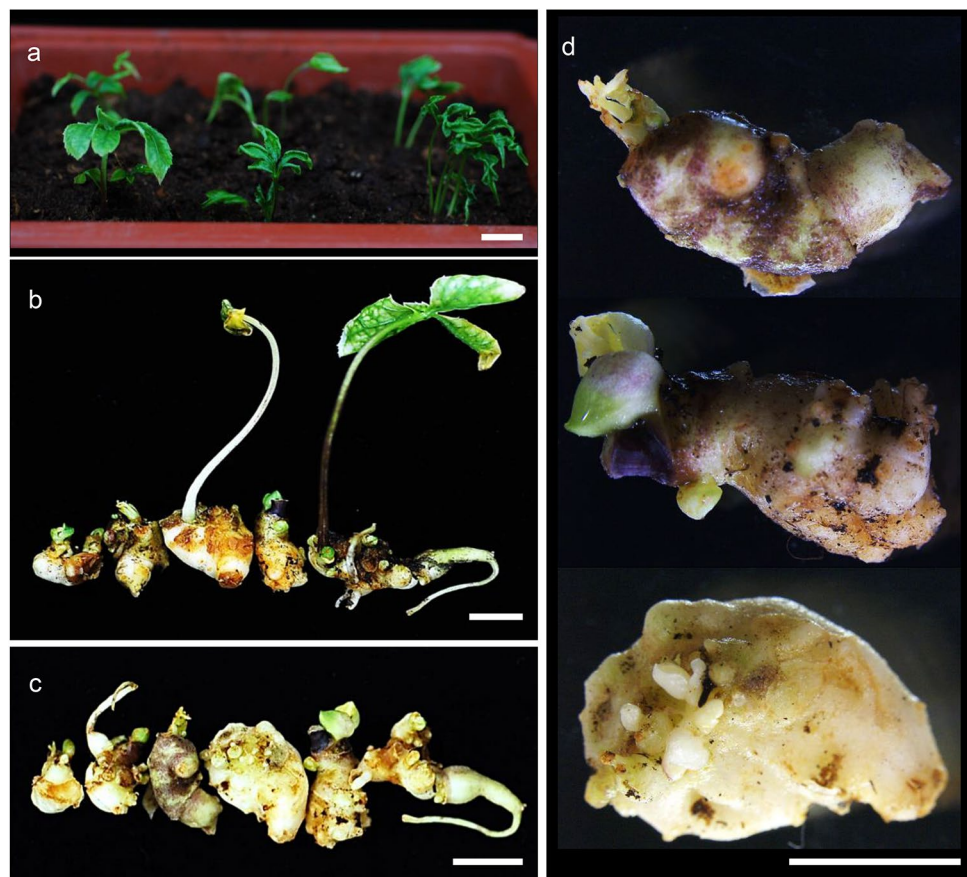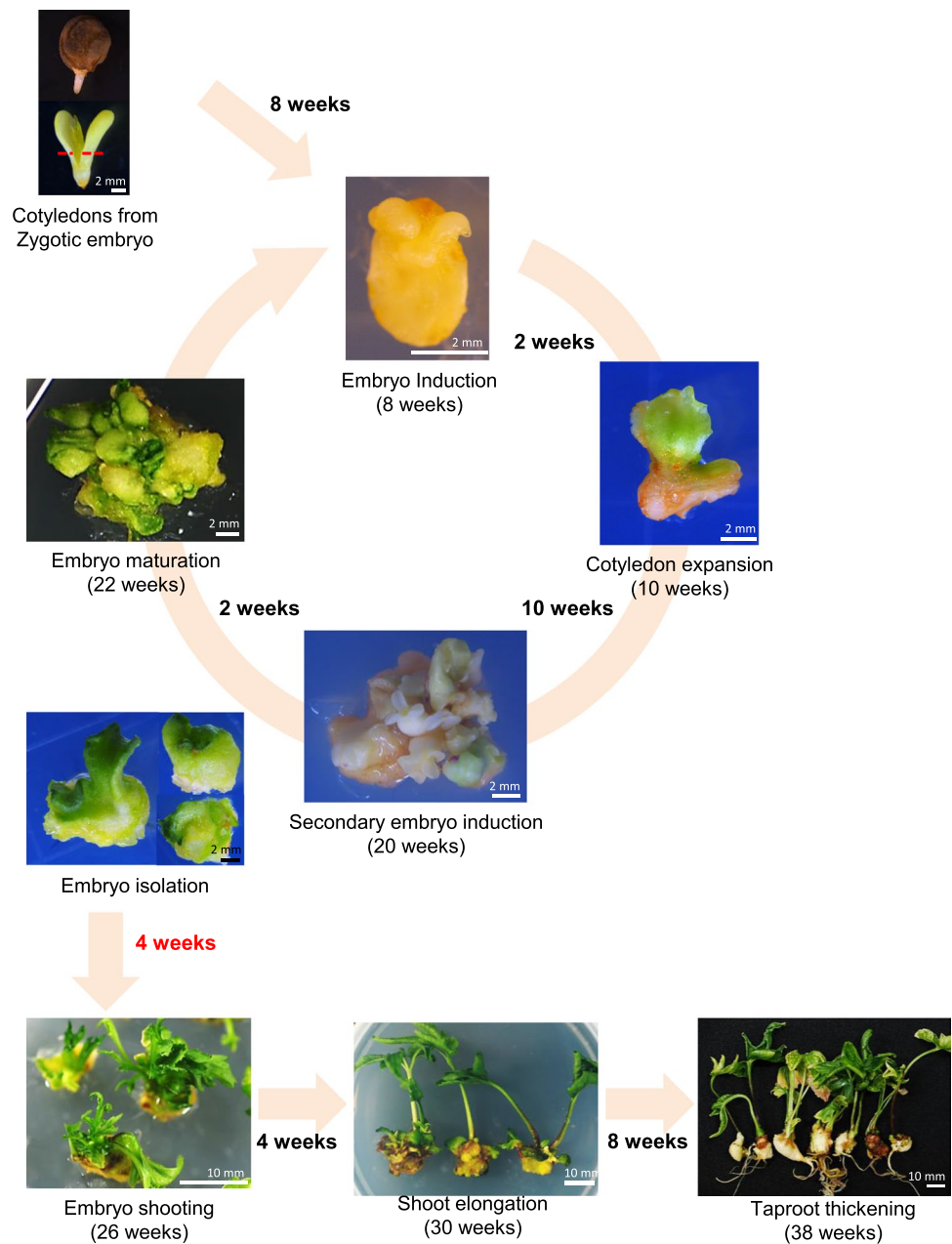
Fig. 5 Establishment of high efficiency an *ex vitro* acclimatized plant production system using secondary somatic embryos in *Panax ginseng* Mayer based on the findings of this study. Different phases of plant regeneration along with a timeline are shown. The primary embryos were induced using cotyledons taken from zygotic embryos. It took a total of 38 weeks to produce regenerated plants with a thickened taproot through embryogenesis that were ready to begin the transplant process to soil

Cotyledons from Zygotic embryo

8 weeks

Embryo Induction (8 weeks)

2 weeks

Cotyledon expansion (10 weeks)

Embryo maturation (22 weeks)

2 weeks

10 weeks

Secondary embryo induction (20 weeks)

Embryo isolation

4 weeks

Embryo shooting (26 weeks)

4 weeks

Shoot elongation (30 weeks)

8 weeks

Taproot thickening (38 weeks)

transplanted into unsterilized peat moss and perlite mixture soil, formed dormant buds on the main roots, and the dormant buds germinated when the dormancy was broken at 4℃ for about 3–4 months. In case of Korean ginseng, the growth condition of the aboveground and underground parts is considered to be a successful acclimatization factor. Taken together, these results can be efficiently utilized to produce successfully acclimatized plants for mass production of Korean ginseng.

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Author contribution ORL conceived the project and designed the experiments. JHK, MYK, and JHL performed the experiments. ORL, JHK, MYK, JHL, and JUK analyzed the data and wrote the paper.

Data availability Data are available upon request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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