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High efficiency plant production of North American ginseng via somatic embryogenesis from cotyledon explants

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Abstract An efficient in vitro protocol for plant production of North American ginseng has been established. The pretreatment of cotyledon explants with 1.0 M sucrose at 4°C resulted in an improvement of embryo quality and, combined with a higher sucrose content (7%) in induction medium, improved the embryogenesis frequency from 40% to 75% and the number of embryos per explant from 10 to 21. The frequency of secondary embryogenesis from somatic embryo-derived tissues cultured on MS medium with 1.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} NAA is up to 90%. Somatic embryos can further develop to maturity on SH medium supplemented with 1% activated charcoal and half of them can germinate. About 85% of the germinated embryos will convert into plants with well-developed taproot systems on ½ SH medium with 0.5% activated charcoal. The growth chamber and field establishment rates were 95.6 and 93.7%, respectively. The plants transplanted to growth chambers and field plots appear normal.

Keywords Embryo quality · Micropropagation · *Panax quinquefolius* · Plant regeneration

Abbreviations AC: Activated charcoal · B_5:Gamborg et al. (1968) · 2, 4-D: 2, 4-Dichlorophenoxyacetic acid · BA: 6-Benzyl-aminopurine · GA_3: Gibberellic acid · MS: Murashige and Skoog (1962) · NAA: α-Naphthaleneacetic acid · SH: Schenk and Hildebrandt (1972)

Introduction

North American ginseng (*Panax quinquefolius* L.) is a native herb of Canada and the U.S.A. and is cultivated for its highly valued root used for medicinal purposes. The plant has a long production cycle, as seeds are usually produced after a 3-year cultivation and must be stratified for an additional 12–18 months before germination. A clonal propagation method based on tissue culture procedures would contribute to its genetic improvement by reducing the generation cycle time by about 80% and allow for the reduction in confounding genoptype effects in field evaluations through the use of clonal material.

Tissue culture of Korean ginseng (*Panax ginseng* C.A. Meyer), a close relative of North American ginseng, is well studied with reports on callus culture (Furuya et al. 1986; Choi et al. 2003), protoplast culture (Arya et al. 1991), shoot organogenesis (Tang et al. 2000; Bonfill et al. 2002), somatic embryogenesis (Chang et al. 1980; Choi et al. 1997; 1998a; 1998b; 1999; Tang et al. 2000; Kevers et al. 2002) and *Agrobacterium*-mediated genetic transformation (Lee et al. 1995; Choi et al. 2001; 2003). There is little evidence that any of these techniques have been used successfully with North American ginseng. There are only a few reports on the in vitro culture of North American ginseng; these documented cases report the formation of somatic embryos on callus culture of embryonic, seedling, root or leaf explants (Wang et al. 1990; 1999; Li et al. 1992; Tirajoh et al. 1998; Brown et al. 2001). Plant regeneration of American ginseng is still considered to be recalcitrant because of the difficulty in obtaining plants with a well-developed root system. Up to now, there is no report on successful transplantation of regenerated plants into the field with either Korean ginseng or North American ginseng.

Few investigations have focused on somatic embryogenesis from cotyledons excised from immature or mature zygotic embryos of North American ginseng, which have proven to be one of the best explant sources for somatic embryogenesis in *Panax ginseng* (Choi et al. 1997; 1998a; 1998b; 1999; 2001; Tang et al. 2000). Using cotyledons as starting explants, a laboratory-scale micropropagation system, based on a nine-stage protocol, was developed for *P. quinquefolius* (Brown et al. 2001). However, the efficiency of plant recovery was low because of the difficulty in obtaining plants with a well-developed root system. The objective of the present investigation

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was to optimize the micropropagation procedure such that tissue-cultured plantlets can be recovered at a high efficiency and successfully transplanted into the field.

Materials and methods

Explant preparation and culture conditions

Explants including cotyledons from stratified and surface-sterilized seeds and somatic embryo-derived tissues, leaf petioles and elongated cotyledons from abnormal regenerated shoots were used to induce somatic embryogenesis. Stratified seeds were taken from the bulk seed grown in simulated commercial plots at the Delhi site of the Southern Crop Protection and Food Research Centre, Delhi, Ontario, Canada. Commercial North American ginseng is usually a collection of land race lines that have not been subjected to controlled genetic selection; thus, there is no accession or specific genetic plant identifier available for the bulk seed source used for this study. This situation would be representative for normal commercial seed used for commercial ginseng production. Seed coats were removed and the naked seeds were surface sterilized by immersing in a 0.1% Benomyl (fungicide, www.sigmaaldrich.com) solution for 15 min, 70% ethanol for 1 min and soaking in a 7% dilution of household bleach for 15 min. The surface-sterilized seeds were rinsed three times with sterile distilled water and zygotic embryos were excised aseptically. Cultures were maintained at 24°C with a 16-h photoperiod of 30 μmol m^{-2} s^{-1} under cool-white fluorescent tubes. All media were solidified with 0.19–0.26% Gelrite (www.sigmaaldrich.com) and autoclaved at 120°C for 20 min. The pH of the media was adjusted to 5.8 before autoclaving.

Induction of somatic embryos

Cotyledons excised aseptically from zygotic embryos were placed abaxial side down on MS (Murashige and Skoog, 1962) basal medium containing 30, 50, 70, or 90 g l^{-1} sucrose with 16 cotyledons per Petri dish. To investigate the effect of sucrose concentration on embryogenesis, an experimental approach was designed to use cotyledon pairs, comparing two concentrations in each experiment. To make the somatic embryos separate or "single," the cotyledons were pre-treated for plasmolysis with 1.0 M sucrose solution at 4°C for 48–168 h (following the steps of Choi et al. 1999). Each treatment had at least three replicates and the experiments were repeated three times. The number of explants producing embryos was recorded at the end of 5th and 7th week of culture (See Fig. 1, Stage 1). For secondary embryo production, elongated cotyledons and leaf petioles were cut from abnormal somatic embryo-derived shoots and placed on MS basal medium supplemented with 1.5 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D) (TR_1), or 1.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} α-naphthaleneacetic acid (NAA) (TR_2), with 25 explants per dish and 8 replicates for each treatment. The number of explants producing embryos was recorded at the end of 5th and 7th week of culture. (See Fig. 1, Stage 4b). Both kinds of explants were cultured in 100×15 mm Petri dishes containing 30 ml of medium and transferred to fresh medium every 2 weeks.

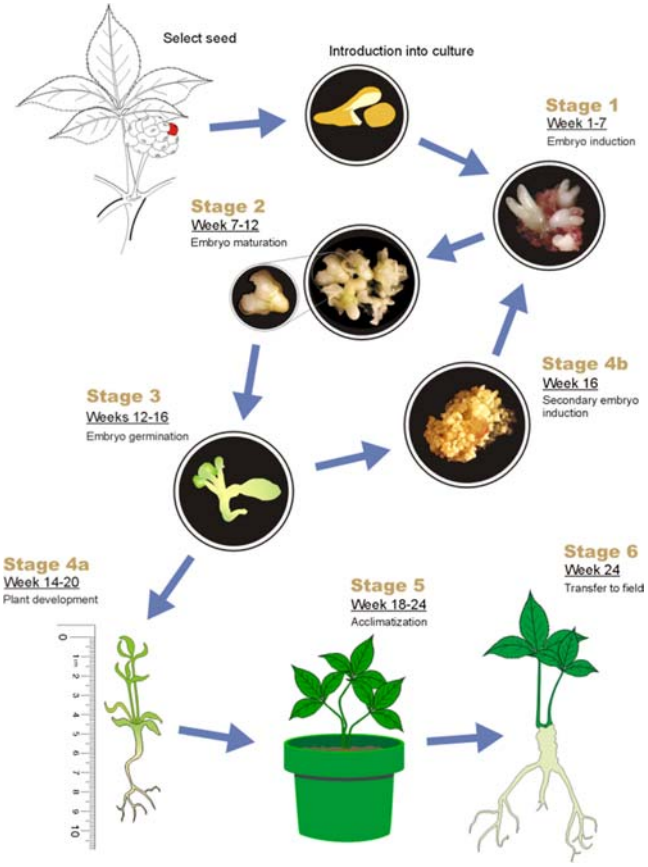

Fig. 1 Flow-chart of the protocol for plant micropropagation of American ginseng. *Stage 1*: Cotyledons from stratified and surface-sterilized seeds are pre-treated with 1.0 M sucrose at 4°C for 72 h (Introduction into culture) and cultured on MS basal medium containing 7% sucrose to induce embryos. *Stage 2*: Cotyledonary embryos are transferred onto SH basal medium containing 1% activated charcoal and cultured for at least 5 weeks. *Stage 3*: Mature embryos are transferred onto SH basal medium containing 10 mg l^{-1} GA_3 and cultured for 2–4 weeks. *Stage 4a*: Germinated embryos are transferred onto ½ SH basal medium containing 0.5% activated charcoal and cultured for 4–6 weeks. *Stage 4b*: Somatic embryo-derived tissues are put on MS basal medium supplemented with 1.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} NAA to induce secondary embryos. *Stage 5*: Plants with a well-developed root system are transplanted into "soil" (Promix BX) to acclimatize. *Stage 6*: Acclimatized plants are transplanted to field

Maturation of somatic embryos

Somatic embryos developed to the cotyledonary stage were transferred from induction media onto maturation media, half-strength MS or SH (Schenk and Hildebrandt, 1972) basal media supplemented with 3% sucrose and 1% activated charcoal, and cultured for 1–2 months (see Fig. 1, Stage 2). Embryos on cotyledon explants were transferred together with the explants, while the secondary embryos

from somatic embryo-derived tissue explants were removed from the embryogenic callus and transferred separately into 100 × 15 mm Petri dishes containing 30 ml medium.

Germination of somatic embryos

Well-developed somatic embryos were transferred from maturation medium onto germination medium, 30 ml MS or SH basal medium supplemented with 10 mg l^{-1} gibberellic acid (GA_3) in 100×20 mm Petri dishes, for germination. The effect of GA_3 concentration on germination was investigated by placing mature somatic embryos on SH basal media containing 2, 5, 10, 20, 30, or 40 mg l^{-1} GA_3, with 40 embryos per dish and 4 replicates in each treatment. The number of embryos germinated with a shoot more than 0.5 cm in length was recorded each week during the first 5 weeks. The number of germinated embryos developing into plants on elongation medium, ½ SH basal medium containing 0.5% activated charcoal was recorded afterwards (see Fig. 1, Stage 3).

Development of plantlets

When shoots reached 0.5–1.0 cm in height, the plantlets were separated with forceps and transferred from germination medium to elongation medium: half-strength MS or half-strength SH basal medium supplemented with 0.5 mg l^{-1} GA_3 and 0.1 mg l^{-1} 6-benzylaminopurine (BA) with or without 0.5% activated charcoal, or just half-strength SH basal medium supplemented with 0.5% activated charcoal. The medium was contained in 70 mm×70 mm×80 mm GA-7 Magenta box, 50 ml per box. Twelve plants/shoots were placed per box. After 4 weeks, the plantlets with a taproot or adventitious roots were transferred into potting mix (See Fig. 1, Stage 4a). Shoots without roots were transferred into rooting medium, half or one-third strength MS, or B_5 (Gamborg et al. 1968) or SH basal medium supplemented with 0.1, 0.25 or 0.5 mg l^{-1} NAA in 20 × 100 mm glass tubes, one shoot per tube. After 1 month, the results of rooting were recorded. The effect of basal medium on development of plantlets was investigated by placing surface-sterilized mature zygotic embryos on MS, B_5 or SH basal medium at full, half and one-third strength, respectively, contained in 20 × 100 mm glass tubes, one embryo per tube and eight embryos for each medium. The experiment was repeated three times. The length of shoots and roots was measured after 3 weeks.

Transplantation of plants

Well-developed plantlets with a taproot or adventitious roots were potted in 65 mm×65 mm×60 mm plastic pots (4 plants per pot) containing moistened potting mix, Promix BX (www.premierhort.com), placed into a 70 mm×70 mm×100 mm GA-7 Magenta box and covered with a second box and a coupler. The potted plants were cultivated in a growth chamber held at 20/16°C (day/night) and a 13-h photoperiod of 120 μmol m^{-2} s^{-1} under cool-white fluorescent tubes. After 2 weeks, the plants were hardened by removing the cover briefly on a daily basis for 2 weeks, and then the cover was removed (see Fig. 1, Stage 5). The acclimated plants were transplanted into field (see Fig. 1, Stage 6) or kept in the chamber for another 3–4 months.

Results and discussion

Somatic embryogenesis

Somatic embryos began to form on the lateral edges and adaxial surface of the cotyledon explants after 4 weeks on MS basal medium without plant growth regulators (Fig. 2a and e). While the percentage of explants with embryos was relatively high, with some explants producing as many as 90 embryos, more than 90% of the resulting embryos were abnormal and fused together (Fig. 2g). The fused embryos gave only multiple shoots, from which quality roots could not be obtained (Brown et al. 2001). This problem in *Panax ginseng* was solved by pre-treating cotyledon explants with 1.0 M sucrose solution for 72 h (Choi et al. 1999). However, when cotyledons of North American ginseng were pre-treated with 1.0 M sucrose solution at room temperature following the same protocol, they all died even with a 12 h treatment. After the failure of pre-treatment at room temperature, cotyledons were pre-treated with 1.0 M sucrose at 4°C. The cotyledons survived even after a 168 h treatment. The pretreatment of cotyledons with 1.0 M sucrose for at least 48 h resulted in significantly more individual somatic embryos and a more uniform formation on the explant surface (Fig. 2b, c and f). The percentage of explants with embryos increased from 40 to 66% (Fig. 3). The average number of embryos on one explant increased from 10 to 21 (data not shown).

North American ginseng is a heterogeneous population (Bai et al. 1997) and the seeds, when first harvested, have immature embryos which mature during the 20-month stratification period. Because of the genetic and physiological heterogeneity of the seeds, cotyledon pairs were used as homogeneous material to obtain accurate experimental results. In the experiment designed with cotyledon pairs, the optimal sucrose concentration in embryogenesis medium was determined to be 7% (Fig. 4). With plasmolysis pretreatment of cotyledon explants and higher sucrose concentration (7%) in induction medium, the percentage of explants with embryos was increased up to 75% (Fig. 4).

The abnormal, fused and small-sized somatic embryos only produce enlarged cotyledons, abnormal and multiple shoots after culture on germination medium. These tissues can be used as explants to produce secondary somatic embryos. Auxins are used to induce embryogenic callus and somatic embryos from these explants. Wang et al. (1999)

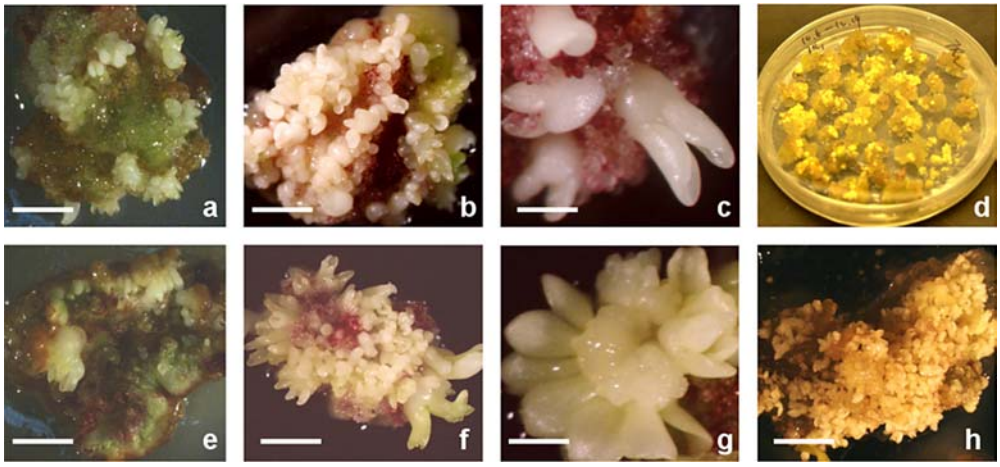

Fig. 2 **a–g** Somatic embryo formation on different explants of American ginseng. **a** and **e** Somatic embryos formed on the lateral edges and adaxial surface of the untreated cotyledon explants. **b** and **f** Uniform formation of individual somatic embryos on cotyledon explants pretreated with 1.0 M sucrose solution. **c** Individual somatic embryos on cotyledon explants pretreated with 1.0 M sucrose solution. **d** and **h** Secondary embryos formed on explants from somatic embryo-derived tissues with a high frequency and a big number. **g** Fused somatic embryos on untreated cotyledon explants. Bars **a**, **b**, **e**, and **f** 2 mm; **c** 0.7 mm; **g** 1.2 mm; **h** 2.5 mm

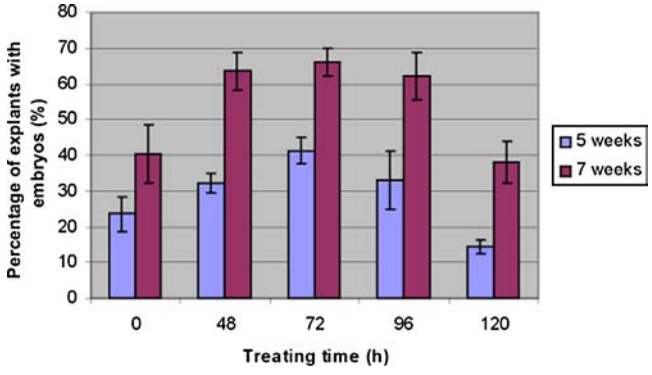

Fig. 3 Effect of plasmolysis pre-treatment on embryogenesis frequency. Cotyledon explants were treated with 1.0 M sucrose at 4°C and cultured on MS basal medium containing 5% sucrose. Vertical bars indicate the standard error of the mean of three replicate experiments

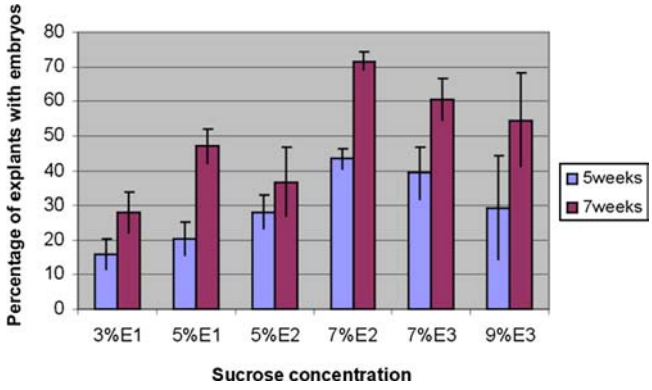

Fig. 4 Effect of sucrose concentration on embryogenesis frequency. The experiment shown was designed to use cotyledon pairs, comparing two concentrations each experiment. E_1, E_2, and E_3 indicate three separate experiments. The cotyledon explants were pre-treated with 1.0 M sucrose at 4°C for 48 h. Vertical bars indicate the standard error of the mean of three replicate experiments

investigated the effects of auxins and explants on somatic embryogenesis of North American ginseng. Their results showed that the optimal concentrations were 1.1 mg l^{-1} 2, 4-D or 2.8 mg l^{-1} NAA, and when used together, the effect of 2, 4-D was independent of the concentrations of NAA. Similar results were obtained in this study on the effect of 2, 4-D, with an optimal concentration of 1.0–1.5 mg l^{-1} (data not shown). However, the combined effect of the two auxins in TR_2 (MS medium containing 1.0 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} NAA) resulted in a higher percentage of explants with embryos compared to medium (TR_1) containing only 2, 4-D (Fig. 5). When NAA was used alone, it gave rise to roots and a much lower embryogenesis frequency (data not shown). On TR_2 medium, the percentage of explants with embryos was 80% (Fig. 5), with up to hundreds of embryos on one explant (Fig. 2d and h), over 90% of which were single and appeared normal. The embryogenic callus can be subcultured on TR_2 for over 6 months and still produce embryos. With the use of this secondary embryo production approach, the efficiency of plant production of the system can be enhanced tremendously.

Maturation and germination of somatic embryos

On induction medium, somatic embryos, when reaching the cotyledonary stage, stopped growing, grew very slowly or began undergoing secondary embryogenesis, while small-size or immature embryos did not germinate into plants. For maturation of the somatic embryos, different basal media in different strengths and with different concentrations of sucrose were tested but none of combinations resulted in development of somatic embryos without activated charcoal. On media (SH or half-strength MS basal medium) containing 1% activated charcoal and 3% sucrose, somatic embryos grew to 4–6 mm in length at maturity in 1–2 months (Fig. 6a, b, and c), providing a prerequisite base for

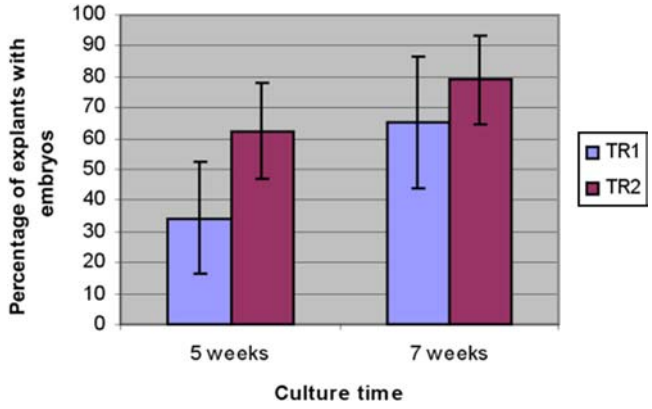

Fig. 5 Percentage of explants with embryos on TR_1 or TR_2 medium. Explants indicates elongated cotyledons or leaf petioles from abnormal shoots derived from somatic embryos formed on cotyledon explants cultured on MS basal medium with 5% sucrose, matured on ½ MS basal medium with 1% activated charcoal and germinated on MS basal medium with 10 mg l^{-1} GA_3. TR_1 MS basal medium supplemented with 1.5 mg l^{-1} 2, 4-dichlorophenoxyacetic acid. TR_2 MS basal medium supplemented with 1.0 mg l^{-1} 2, 4-dichlorophenoxyacetic acid and 1.0 mg l^{-1} α-naphthaleneacetic acid. Vertical bars indicate the standard error of the mean of eight replicate experiments

strong plants. After 1–2 months on maturation medium, the well-developed embryos were transferred onto germination medium, SH or MS basal medium with GA_3 and germinated in 2–4 weeks (Fig. 6d, e, and f). The optimal concentration of GA_3 in germination medium was 10–20 mg l^{-1}. A lower concentration (i.e., 5 mg l^{-1}) of GA_3 gave a much lower germination frequency. A higher concentration (i.e., 30–40 mg l^{-1}) of GA_3 resulted in a weak and abnormal shoot and a subsequent low conversion rate of plantlets. About 54% of the embryos can germinate to form normal shoots on this germination medium (Table 1).

Development and transplantation of plants

Germinated embryos with normal shoots (0.5–1.0 cm high) obtained from the above-described optimal germination medium were transferred to elongation medium (Fig. 7a). When transferred from germination medium to elongation medium, most of the germinated embryos had no visible taproots, and the taproots did not develop in any of the tested elongation media without activated charcoal. However, in half-strength SH basal medium supplemented with

Table 1 Effect of GA_3 concentration on germination of somatic embryos and conversion of germinated embryos [a]

Concentration (mg l^{-1}) of GA_3 [b]	Cumulative germination (%) at various times (weeks)[c]				Conversion (%±SE) to plantlets in 4 weeks [d]
	2	3	4	5	
5	0	12.00	12.00	16.67±5.46	–
10	12.63	26.32	44.21	52.70±0.63	83.81±2.35
20	12.86	32.86	47.14	56.04±1.35	85.41±2.24
30	10.96	30.97	41.29	52.36±4.41	74.40±2.91
40	9.68	29.68	47.10	53.45±1.72	63.84±5.84

[a] Secondary embryos produced from somatic embryo-derived tissues cultured on TR_2 medium (see methods)

[b] Basal medium was SH

[c] Germination was based on the presence of shoots more than 0.5 cm in length

[d] Plantlets from germinated embryos developed on half-strength SH basal medium supplemented with 0.5% activated charcoal

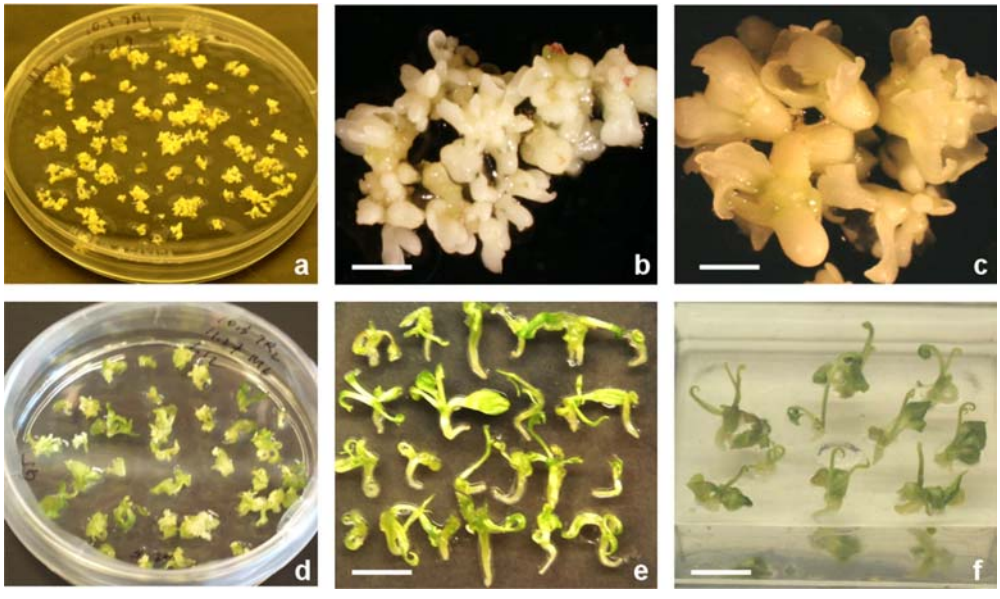

Fig. 6 a–f Maturation and germination of somatic embryos. **a**, **b**, and **c** Development of somatic embryos on maturation medium, SH basal medium containing 1% activated charcoal. **d**, **e**, and **f** Germination of mature embryos on germination medium, SH basal medium supplemented with 10 mg l^{-1} GA_3. Bars **b** and **c** 2 mm; **e** 0.9 cm; **f** 1.2 cm

Fig. 7 **a–f** Development of plants and transplantation into soil and field. **a** Germinated embryos transferred onto elongation medium. **b** Plants developed 4 weeks on elongation medium, ½ SH basal medium containing 0.5% activated charcoal. **c** Plants with a taproot developed on elongation medium, ½ SH basal medium containing 0.5% activated charcoal. **d** Rooted plants on rooting medium, 1/3 SH basal medium supplemented with 0.25 mg l^{-1} NAA. **e** Two-month old potted plants. **f** Three-month old plant just before transplantation to the field, showing a thickened root system and a dormant bud

0.5% activated charcoal, about 85% (Table 1) of the germinated embryos developed into plants with well-developed taproots (Fig. 7b and c). After 1–2 months on the elongation medium, well-developed plants were transplanted into potting mix (Promix BX). The remaining shoots without roots were transferred to rooting medium to root. One-third strength SH basal medium supplemented with 0.25 mg l^{-1} NAA gave the best result (Fig. 7d and Table 2).

The effect of basal medium on root development can be seen from Table 2. In general, half-strength MS medium has been used for plantlet development, but in the medium, even the plantlets derived from single embryos with both shoots and roots, the roots grow much slower than shoots, and even stop growing and turn brown. A similar problem was reported in *Panax ginseng* (Choi et al. 1997; Choi et al. 1998), where MS medium with ammonium nitrate omitted was used to solve this problem. It seems that a high level of ammonium nitrate in the nutrient medium is good for somatic embryogenesis of ginseng but detrimental to root development as was demonstrated in carrot (Halperin 1966). The results from the experiments testing the effect of basal medium on plant development of North American ginseng conducted with zygotic embryos supports this inference. When zygotic embryos of North American ginseng were cultured on full, half or one-third strength MS medium, they grew very little after germination and gradually browned and eventually died. B_5 medium at the same three levels of strength gave much better results, while the best results were observed on SH medium, especially on one-third strength SH (Fig. 8). In one-third strength SH medium, the shoots grew strongly and the roots grew fast and thickened as they would in soil and with normal color.

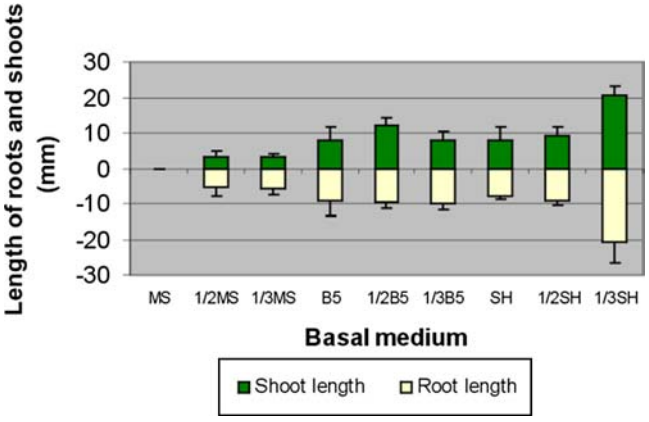

Fig. 8 Effect of basal medium on growth of seedlings of American ginseng. Vertical bars indicate the standard error of the mean of three replicate experiments

Table 2 Comparison of rooting media. Data represents the results obtained from shoots developed on ½ MS basal medium supplemented with 0.5 mg l^{-1} GA_3 and 0.1 mg l^{-1} BA

Media	No. of shoots	Rooting frequency (%)	No. of roots per plant	Description on root quality
½ MS + 2% Sucrose + 0.1 mg l^{-1} NAA	41	22.0	–	Grows very slow and turns brown
½ MS + 2% Sucrose + 0.5 mg l^{-1} NAA	30	30.0	–	Grows very slow, calluses and browns
½ B_5 + 2% Sucrose + 0.25 mg l^{-1} NAA	33	54.5	2.4	Grows slowly
½ B_5 + 2% Sucrose + 0.5 mg l^{-1} NAA	30	50.0	1.3	Grows slow and calluses
½ SH + 1.5% Sucrose + 0.25 mg l^{-1} NAA	26	57.7	3.1	Grows fast
1/3 SH + 1% Sucrose + 0.25 mg l^{-1} NAA	40	80.0	3.8	Strong and grows fast

One of the most distinct differences among the three basal media is the concentration of NH_4^+ ion, 20.6 mM in MS, 2.0 mM in B_5 and 2.6 mM in SH. It seems that the high concentration of NH_4^+ ion in MS medium inhibits the development of ginseng plants and specifically roots. SH medium as basal medium has successfully been used in a bioreactor system for the adventitious roots of *Panax ginseng* (Choi et al. 2000). Because of the above observations, we suggest that SH basal medium be used, after induction of somatic embryos, for maturation and germination of somatic embryos and elongation of plantlets in ginseng.

Of over 600 plants that were transplanted into potting mix (Promix BX), 200 plants were transplanted into the field at various times during the summer season of 2004. The growth chamber and field establishment rates, 5 weeks after transplantation, were 95.6 and 93.7%, respectively. The transplanted plants appear normal and grew well (Fig. 7e), showing the development of a dormant bud and a thickened root structure typical of seed-derived plants (Fig. 7f).

In conclusion, the efficient protocol described here allows for a consistent production of North American ginseng plants and a very high field establishment rate (94%). It takes 24 weeks from the introduction into culture of cotyledons to the transplantation of plants to the field (Fig. 1). Approximately 26 [2 (cotyledons) × 65% (percentage of cotyledons with embryos) × 20 (embryos per cotyledon)] embryos can be produced from two cotyledons (one seed) in 7 weeks (Fig. 1 Stage 1) and about 674 [26 (embryos) × 54% (germination rate) × 3 (explants each embryo) × 80% (percentage of explants with embryos) × 20 (embryos per explant)] embryos can be produced through secondary embryo production in another 7-week period (Fig. 1 Stage 4b). Finally about 310 [674 (embryos) × 54% (germination rate) × 85% (conversion rate)] plants from one stratified seed can be produced in about 31 weeks. In comparison, one stratified seed produces about 30 plants in a conventional 166-week production cycle. The protocol could possibly be further improved by using immature seeds selected from plants in the field without stratification or by using mature explants from young field-grown plants. This technique with North American ginseng provides the basis for a relatively rapid multiplication of selected genotypes and will allow a much more rapid evaluation of the germplasm of the unexplored and unstudied North American ginseng.

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