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Rapid Somatic Embryogenesis and Plant Regeneration in American Ginseng: Effects of Auxins and Explants

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Abstract : The efficacy of three auxins, viz. 2,4-D, NAA and dicamba, were compared for the induction of somatic embryogenesis in American ginseng *(Panax quinquefolium* L.). Somatic embryos (SEs) formed on ginseng cotyledonary, zygotic embryo and shoot explants after 8 weeks of induction by the auxin stimuli. Significantly more somatic embryos were induced by culture of any of the ginseng explants on media supplemented with 5 μM 2,4-D than any other auxin treatment. Shoots derived from somatic embryos had the greatest regenerative potential and zygotic embryos the least. Explants generated from green (unstratified) seeds gave similar or higher frequency of embryogenesis as the explants derived from stratified seeds. Histological and SEM studies confirmed that the regenerants were somatic embryos. Somatic embryos germinated and developed into normal plants in 3~6 months. About 10% of plantlets from second generation SEs formed flowers within 10 weeks, particularly on media supplemented with GA_3. The development of a regeneration system for ginseng through somatic embryogenesis is a necessary first step for mass propagation and genetic improvement of American ginseng.

Key words : somatic embryogenesis, auxin, American ginseng, naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid.

Introduction

Ginseng is a very valuable agricultural species prompting it to be called green gold (Persons, 1986). For thousands of years ginseng has been used in Chinese medicine as a wonder drug, cure-all, and aphrodisiac (But *et al.*, 1995). Two species of ginseng are currently of commercial importance: Oriental ginseng (*Panax ginseng* C. A. Meyer) which is native to northeastern China and Korea, and American ginseng (*Panax quinquefolium* L.) which is native to parts of eastern Canada and the U.S.A., from Quebec to Louisiana, Alabama and Arkansas (Oliver *et al.*, 1992). One limiting factor for the future expansion of ginseng production is an efficient method for mass propagation. Currently, seeding is the principal method of propagating ginseng (Proctor and Bailey, 1987) but the embryo of ginseng seeds at harvest is immature (Stoltz and Snyder, 1985). This immaturity of the embryo delays the germination of ginseng seeds in the field and makes ginseng seeds susceptible to adverse environmental conditions, and disease. In general, a stratification schedule consisting of a cool-warm-cool temperature treatment over a 18~22 months is required for embryo development and seed germination (Proctor and Louttit, 1995).

An alternative for both efficient production and the improvement of ginseng crops is mass propagation through the use of *in vitro* culture techniques. Somatic embryogenesis has been described in many plant species since the first reports in carrot (Steward *et al.*, 1958; Reinert, 1958). Butenko *et al.* (1968) first reported induction of *in vitro* somatic embryos from root and embryo-derived callus cultures of ginseng. Later, callus and somatic embryos were developed from zygotic embryos (Gui *et al.*, 1987; Li and Guo, 1990), epicotyls, leaves (Tirajoh and Punja, 1995), and roots (Wang, 1990;

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Tirajoh *et al.*, 1998) of American ginseng.

In most of the regeneration systems currently described for ginseng, somatic embryogenesis is induced by an exogenous auxin. The synthetic auxin 2,4-D has been used in both Oriental (Chang and Hsing, 1980; Jhang *et al.*, 1974; Kishira *et al.*, 1992) and American ginseng (Gui et al., 1987; Li and Guo, 1990; Jhang *et al.*, 1974), as well as in *Panax notoginseng* (Shoyama *et al.*, 1997). Similarly, somatic embryos have been obtained from leaf explants of *in vitro* grown seedling after 3 months of culture on 2,4-D and NAA supplemented medium (Tirajoh and Punja, 1995). Recently, Tirajoh *et al.*, (1998) reported the induction of embryogenic callus derived from root, leaf and epicotyl explants of American ginseng cultured in the presence of auxins, NAA, 2,4-D, or dicamba. In addition, somatic embryogenesis has been induced from root derived callus by supplementation of the culture media with dicamba (Wang, 1990). However, to date, no comparative study of the effect of different types of auxins and their concentrations on somatic embryogenesis in American ginseng has been reported.

Therefore, the overall goal of this study was to develop a highly efficient system for regeneration of ginseng. The specific objectives of this study were: a) to establish the optimal level and type of auxin for the induction of somatic embryos from zygotic embryo-derived and somatic embryo-derived explants, b) to compare the effects of different auxins and their combinations on somatic embryogenesis in various types of explants, and c) to optimize the culture conditions for somatic embryo germination and plantlet regeneration.

Materials and Methods

Stratified seeds (mixed with sand and stored in sandbox outdoor for one year; Proctor and Louttit, 1995) were obtained from commercial growers in Waterford, Ontario. Seeds were size graded, and extra large (>5.95 mm diameter) and extra small (<5.16 mm diameter) seeds were removed. The remaining seeds were mixed with moist sand, and stored in a cooler (3±0.2°C). Green seeds, that did not have the traditional stratification process were purchased in the Fall, graded and mixed with moist sand as described above, and then stored at 15±0.2 °C for 3 months before transferring to 3°C until used (Hovius, 1996). Seeds with cracked seed coat were removed from the cooler, washed and surface sterilized by immersion in 40% commercial bleach solution (2.2% sodium hypochlorite) with Tween 20 (two drops per 100 ml solution) for 10 min with continuous stirring. The seeds were then rinsed for 3 to 5 times with sterile distilled water, and the seed coat was removed. Seeds were then immersed in 70% ethanol for 2 min, and re-sterilized with 2.2% sodium hypochlorite for 20 min with continuous stirring, followed by at least 5 washes with sterile distilled water.

Three types of explants were compared for embryogenic response; 1) zygotic embryos-intact mature embryos (4~5 mm long) excised from stratified seed, 2) cotyledons-isolated cotyledons of *in vitro* grown seedlings and 3) shoots-shoots obtained from somatic embryos.

1. Zygotic embryo germination

For *in vitro* germination of ginseng zygotic embryos, the endosperm of the seeds was sliced aseptically under a dissecting microscope and the zygotic embryo was excised. Embryos were cultured on a medium containing 1.0 μM gibberellic acid (GA_3), MS (Murashige and Skoog, 1962) salts, B_5 vitamins (Gamborg *et al.*, 1968) and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.6 and gelrite (0.25%) (Scott Laboratories, Carson, USA) was used as the gelling agent. The medium was autoclaved at 1.19 kg cm^{-2} for 20 min. Cultures were maintained in a growth room at 24°C under 16 h light period (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) cool white fluoescent lamps (Philips, Scarborough, Ontario) for one week.

2. Callus and somatic embryo induction

The frequency of somatic embryo induction was compared by culturing zygotic embryo or cotyledonary explants on basal medium supplemented with either 2,4-D (1, 5, 10, 15 μM), NAA (5, 10, 15 μM) or dicamba (5, 10, 15 μM). Somatic embryo-derived shoot explants were cultured on the same NAA and dicamba supplemented medium but the concentrations of 2,4-D were modified to 1, 2, 5 or 8 μM. In a second series of experiments designed to test the effect of culture duration on 2,4-D containing medium, zygotic embryo, cotyledonary, and shoot explants were maintained on media containing 5 μM 2,4-D for 0, 2, 4, 6, or 8 weeks. The final series of experiments was designed to test the effect of combinations of 2,4-D (5, 10, and 15 μM) and NAA (5, 10,

and 15 μM) on induction of somatic embryogenesis in zygotic embryo, cotyledonary tissue, seedling segment and shoot explants of ginseng.

In most experiments, four explants were placed in each Petri dish with ten dishes (replicates) for each treatment, and each experiment was repeated at least twice.

Cultures were examined weekly, and the time required for somatic embryo induction represents the period preceeding the emergence of regenerants. Embryogenic frequency (percentage of explants with somatic embryos) and the number of somatic embryos produced in each dish were recorded after 8 weeks of induction. A chi-square test was used to obtain the trend analysis for the embryogenic frequencies produced with different levels of growth regulators. Log transformation was done on the number of somatic embryos and the analysis of variance was performed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS Institute, 1985). The occurrence of adventitious roots and bud-like structures was also recorded.

3. Light and scanning electron microscopy

Callus with somatic embryos were harvested and cut into pieces of about 0.4 cm^2 and fixed in FAA (50% ethanol: 10% formalin:glacial acetic acid; 18:1:1) for 24 h at room temperature. The explants were then dehydrated in a graded series of ethanol (50, 70, 80, 95, and 100%), and embedded in paraffin as described by OBrien and McCully (1981). Ten μm thick sections were cut with a microtome (Spencer 820, American Optical Co., New York), mounted on glass slides with Haupts adhesive, and placed on a warm tray (27~30°C) for 1-2 d to allow the sections to expand. The process for deparaffinizing was similar to that outlined by Johanson (1940). Sections were passed through 100% Hemo-De, 50:50 100% Hemo-De:100% ethanol followed by a graded series of ethanol. The specimens were then stained with Alcian Green/Safranin Alcian for 30 min, rinsed with water, then put through a series of ethanol from 50, 70, 90, 95, to 100%, and eventually to 100% Hemo-De. Permanent slides were made with Eukitt. Representative sections were photographed with a Zeiss Universal photomicroscope (Carl Zeiss, Oberkochen, Germany).

Sample preparation for scanning electron microscopy was as described by Massicotte *et al.*, (1987). Somatic embryos at various developmental stages were isolated from callus tissue, and fixed with 4% glutaraldehyde in 0.07 $molL^{-1}$ phosphate buffer at pH 6.8 for 3~24 h at room temperature with occasional shaking (Massicotte *et al.*, 1987). Glutaraldehyde was removed and the somatic embryos were washed twice in phosphate buffer, 5 min each, dehydrated in a graded ethanol series, 30% for 90 min, then 50, 70, 90, 95, and 100% each for 30 min. The 100% ethanol was changed twice, one hour for each time, and then the samples were stored in 100% ethanol at room temperature until used. Dehydrated samples were critical-point dried, mounted on stubs with two-sided tape, sputter coated to 30 nm thickness with gold / palladium (Anatech, Hummer VIII, Alexandria, VA), and examined under a JEOL JSM-35c or Hitachi S-570 (Tokyo) scanning electron microscope.

4. Plantlet regeneration

(1) First generation somatic embryos (SEs)

In one set of experiments, four-month-old SEs obtained from stratified seed were used. Individual SEs were collected from the callus tissues under a dissecting microscope and cultured in Petri dishes. Two types of media, viz., a) half strength MS salts, B5 vitamins, 1.5% sucrose, and 3% gelrite (BM) plus 1.45 μM GA_3; b) BM plus 1.45 μM GA_3 and 2.2 μM BAP were tested. In addition, four-month-old SEs obtained from green seeds were also used. The following specific experiments were carried out: a) zygotic embryos (ZEs) of green seeds were cultured on 1 or 3 μM GA_3 enriched MS medium under dark or light (16-h photoperiod); b) excising the two-week-old seedlings into 5 mm long segments, then cultured on 5 μM 2,4-D and 15 μM NAA supplemented MS medium for 4 months; c) individual SEs were collected and cultured on BM supplemented with 1.45 μM GA_3. The following parameters were used for comparison of the effect of growth media on SE growth after 4 weeks of culture: 1) conversion rate, which is the percentage of SEs forming plantlets (having both shoots and roots) among the total number of SEs cultured; 2) percentage of SEs that formed shoots only; and 3) percentage of other SEs, that included small SEs which did not develop further, SEs having elongated radicle only and green structures with no identified shoots or roots.

Plantlets developed from stratified seed-derived SEs were subcultured onto BM after 4 weeks of culture. After 10 weeks of culture, the plantlets were removed

from the medium, the agar rinsed out with sterile distilled water, and planted onto sterile Jiffy 7 pellets. The transplanted plantlets were placed in magenta culture vessels with a cover, watered with 1/4 MS salts, and kept in a growth chamber in 16 h/8 h (light/dark) photoperiod and 22/16°C (day/night). After one week of transferring, the covers of the magenta boxes were removed, but the boxes were kept in a green tray with clear plastic cover. The plantlets (with Jiffy pellets) were transferred into plastic pots with Promix-BX after about one month, and kept in the growth chamber under the same light and temperature conditions. Growth parameters including height of shoots and roots, number of shoots, diameter of root, and number of leaflets per shoot were recorded on 4-month-old plantlets.

(2) Second generation somatic embryos (SEs)

The secondary generation SEs were cultured on four different culture media: a) BM alone; b) BM supplemented with 1.45 μM GA_3; c) BM plus 1.45 μM GA_3 and 2.2 μM BAP; and d) BM containing 2.9 μM GA_3 and 4.4 μM BAP. After 4 weeks, the SEs were transferred to a medium without the growth regulator(s). The frequency (%) of SE conversion into plantlets, shoots and others was recorded after 4 weeks of culture. Growth parameters and in vitro flowering were recorded after 10 weeks of culture. The plantlets developed after 10 weeks of culture were removed from the medium, and transferred to the greenhouse as described for first generation somatic embryos. Growth parameters including height of shoots and roots, number of shoots, diameter of root, and number of leaflets per shoot were recorded on 4-month-old plantlets.

Results

1. Auxin type and concentration

(1) Cotyledonary explants

The highest frequency of embryogenesis on cotyledonary explants was on medium containing 5 μM 2,4-D (43%) or 15 μM NAA (47%) (Table 1). Explants cultured on media containing 10 or 15 μM 2,4-D formed a dark yellow callus and a few small somatic embryos in the globular stage were observed. In addition, 1 to 10 roots were observed per explant cultured on medium containing NAA and in general, more roots were found with higher auxin concentrations. On media supplemented with dicamba, the highest frequency (25%) of embryogenesis was at 5 μM and the frequency of regeneration declined as the concentration of dicamba increased. No regeneration was observed on media devoid of growth regulators (controls).

(2) Zygotic embryo explant

Callus formed on cotyledons and on the base of the germinating zygotic embryos cultured on 2,4-D con-

Table 1. Effect of 2,4-D, NAA, and dicamba on somatic embryogenesis in cotyledonary, zygotic embryo and shoot explants of American ginseng after 8 weeks of induction.

Treatment	Frequency (%)			Number of Somatic Embryos per Plate		
	Cotyledon	Zygotic Embryo	Shoot	Cotyledon	Zygotic Embryo	Shoot
Control[Z]	0	0	0	0	0	0
1 μM 2,4-D	23.7±6.2	5.6±5.6	54.2±8.0	1.5±0.7	0.1±0.1	20.8±2.1
5 μM 2,4-D	43.4±6.6	12.5±72	52.1±11.3	4.2±0.8	0.9±1.0	9.1±2.1
10 μM 2,4-D	25.0±5.4	11.1±4.4	35.4±8.4	1.9±0.8	0.6±0.8	4.7±1.4
15 μM 2,4-D	14.5±4.8	5.0±3.3	20.8±9.2	0.7±1.0	0.2±0.1	1.4±1.1
Trend[Y]	Q	Q	Q	Q	Q	Q
5 μM NAA	13.2±3.5	2.8±2.8	50.0±6.2	0.7±0.9	0.1±0.0	10.2±1.7
10 μM NAA	37.5±6.4	7.5±5.3	56.3±10.7	2.7±0.7	0.2±0.2	12.5±2.6
15 μM NAA	47.4±5.4	22.2±5.0	58.3±9.9	8.3±0.9	1.5±0.6	15.2±2.5
Trend[Y]	L	L	Q	L	L	Q
5 μM dicamba	25.0±5.1	0	45.0±13.3	1.5±0.6	0	4.5±1.9
10 μM dicamba	12.5±4.3	3.6±3.6	15.0±4.1	0.5±0.6	0.1±0.0	1.6±1.0
15 μM dicamba	5.0±2.3	3.3±3.1	13.9±9.4	0.2±0.1	0.1±0.0	0.4±0.9
Trend[Y]	Q	NS	Q	Q	NS	Q

[Z]Basal media containing no plant growth regulators

[Y]Significant p=0.05, L=linear, Q=quadratic, NS=not significant

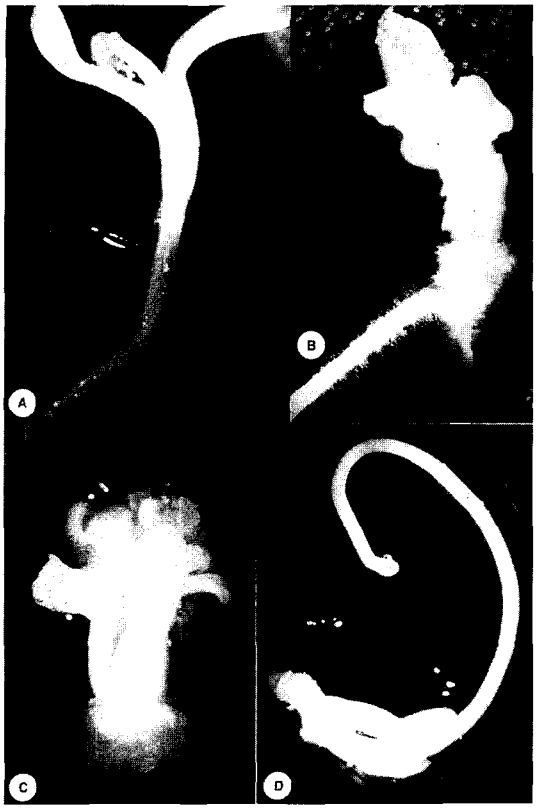

Fig. 1. Zygotic embryo explants after 5 weeks of culture. A. Seedling grown on basal medium without growth regulators (4.5x). B. Adventitious roots developed on explants cultured on 10 μM NAA enriched medium (4.3x). C. Callus formed from explants cultured on medium supplemented with 5 μM 2,4-D (4.3x). D. Seedling with elongated shoot resulting from exposure to 5 μM dicamba in the culture medium (2.8x).

taining medium (Fig. 1). Supplementation of the medium with 5 μM 2,4-D resulted in the highest frequency (12 %) of SE induction among the concentrations tested (Table 1). Callusing, adventitious root initiation and somatic embryogenesis were observed on the zygotic embryos cultured on NAA containing medium and the highest frequency of regeneration was 22% at 15 μM. Dicamba enriched medium had the lowest regeneration frequency (0~3%): the somatic embryos formed elongated and curled shoots (Fig. 1).

(3) Shoot explants

Low concentrations of 2,4-D (1 and 2 μM 2,4-D) induced the highest frequency (52~54%) of somatic embryos (Table 1). At higher concentrations of 2,4-D, shoot explants formed callus which had a watery-appear-

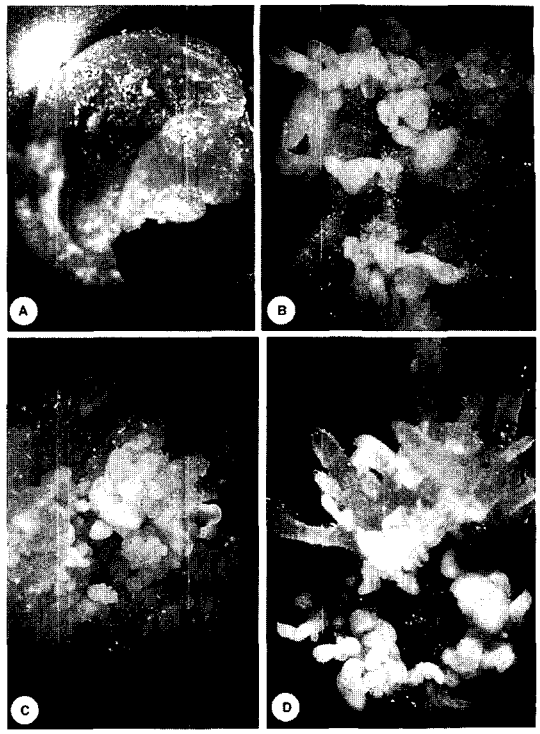

Fig. 2. Shoot explants after 8 weeks of culture. A. Brown explant cultured on basal medium (8.5x). B. Somatic embryos induced by culture on medium containing 1 μM 2,4-D (6.4 x). C. Somatic embryos induced by supplementation of the culture medium with 8 μM 2,4-D (6.4x). D. Somatic embryos and adventitious roots induced by supplementation of the culture medium with 10 μM NAA (6.3x).

ance, with a few small somatic embryos (Fig. 2). Adventitious roots were observed on explants cultured on NAA containing medium, however high frequencies (50~58 %) of somatic embryo induction were observed for all NAA concentrations tested (Table 1). Five μM dicamba gave the highest frequency (45%) of regeneration in shoot explants exposed to this auxin.

In general, the response to 2,4-D and dicamba exhibited a similar quadratic trend with the optimal level around 5 μM except in the case of shoot explants, in which 1 or 2 μM 2,4-D resulted in significantly more regeneration than either 5 or 8 μM. The regenerative potential of explants cultured on media supplemented with NAA increased linearly as the concentration increased from 5 to 15 μM. Among the 3 types of explants, shoots derived from somatic embryos had the greatest regenerative potential and zygotic embryos the least.

2. Effect of exposure period to auxin containing medium

(1) Cotyledonary explants

Somatic embryogenesis was observed on 34~47% of explants exposed to the established optimal level of 2,4-D (Table 2). An exposure time of 4 or 6 weeks gave a significantly higher frequency of regeneration than either a 2 or 8 week exposure. Adventitious roots were observed in 50% of the explants which had either 2 or 4 weeks of exposure to 2,4-D, and the somatic embryos which formed on explants after 2 weeks exposure to 2,4-D often appeared fused together (Fig. 3B). Explants which were maintained on 2,4-D containing medium for the entire 8 week period had somatic embryos at various stages of development (Fig. 3C).

(2) Zygotic embryo explants

About 90% of the explants which had no exposure to 2,4-D germinated with the seedling length up to 50 mm, but no callus or somatic embryos were observed. Embryogenesis was observed on only 2.6% of the explants following a 2 week exposure to 2,4-D, which was significantly lower than the frequency (10~16%) observed with longer exposures (Table 2).

(3) Shoot explants

Although most explants which had no exposure to 2,4-D were brown in colour, 4.2% of the shoot explants formed somatic embryos in the absence of any exogenous plant growth regulators (Table 2, Fig. 4A). Supplementation of the media with 2,4-D for 2~8 weeks induced an embryogenic frequency of 52~68%, and up to 42 somatic embryos/plate were observed (Table 2). Some of the somatic embryos that formed on shoot explants after 2 weeks of exposure to 2,4-D were fused and 39~56% of the explants which had 2~6 weeks of

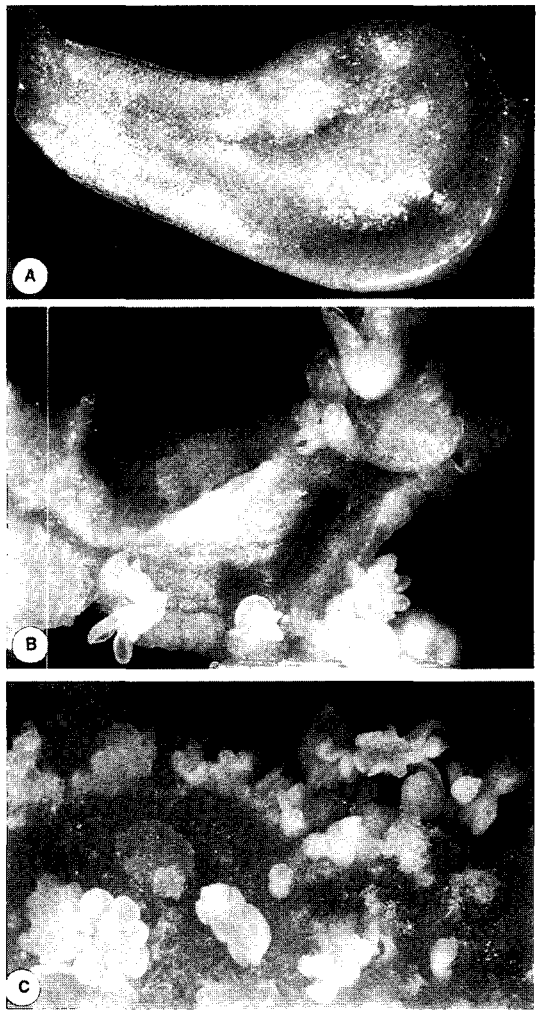

Fig. 3. Somatic embryo induction in cotyledonary explants cultured on 5 μM 2,4-D supplemented MS medium for various times. A. Brown explant which had no exposure to 2,4-D (6.8x). B. Somatic embryos observed on explants after 2 weeks of exposure to 2,4-D (6.8x). C. SEs produced on explants after 8 weeks of exposure to 2,4-D (6.8x).

Table 2. Effect of exposure time to 5 μM 2,4-D on induction of somatic embryogenesis in cotyledonary, zygotic embryo and shoot explants of American ginseng.

Treatment (weeks)	Frequency (%)			Number of Somatic Embryos per Plate		
	Cotyledon	Zygotic Embryo	Shoot	Cotyledon	Zygotic Embryo	Shoot
0	0	0	4.2±2.8	0	0	0.3±0.7
2	34.2±6.1	2.6±1.8	62.5±9.0	3.4±0.8	0.1±0.0	21.4±3.2
4	47.2±7.8	10.5±5.5	68.8±8.2	6.2±1.2	0.4±0.7	42.3±6.1
6	44.4±7.2	15.8±4.4	66.7±7.7	4.4±1.0	1.0±0.7	39.5±4.7
8	34.2±6.1	13.2±2.4	52.1±8.4	3.6±0.8	0.5±0.6	30.3±4.4
Trend[z]	Q	L	Q	NS	Q	Q

[z] Significant p=0.05, L=linear, Q=quadratic, NS=not significant.

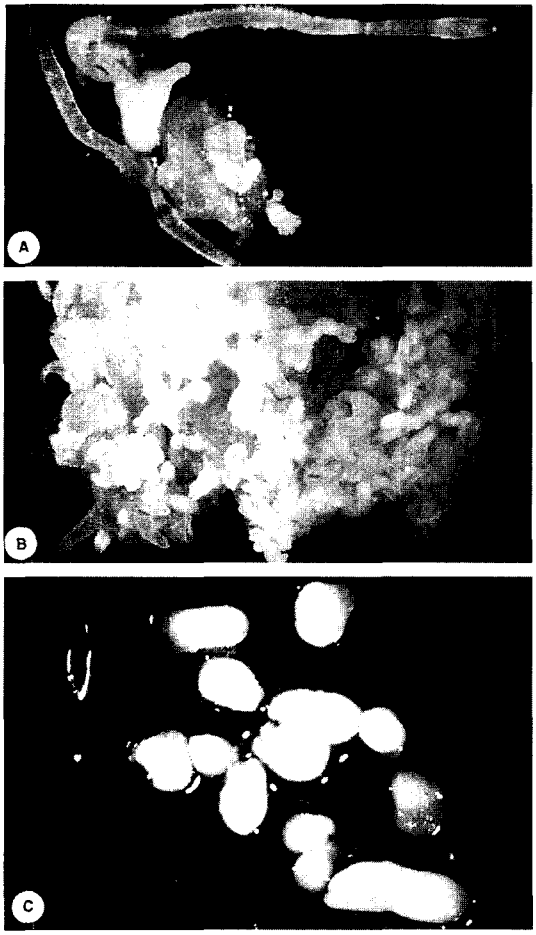

Fig. 4. Somatic embryo (SE) induction in shoot explants cultured on 5 μM 2,4-D supplemented medium for various times. A. A few SEs and adventitious roots occurred on explants which had no exposure to 2,4-D (4.3x). B. SEs produced on explant had 4 weeks of exposure to 2,4-D (4.3x). C. SEs produced on shoot explants after 8 weeks of exposure to 2,4-D (12.8x)

exposure to 2,4-D formed adventitious roots (Fig. 4).

(4) Combined effects of 2,4-D and NAA

Concentrations of 2,4-D (5, 10, 15 μM) significantly influenced embryogenesis in all the somatic embryo-derived explants tested, and the effect of 2,4-D was independent of the concentrations of NAA. In cotyledonary explants, about 58% of the explants cultured on 5 μM 2,4-D supplemented medium in the presence of NAA gave rise to somatic embryos (Table 3, Fig. 5A, B). The three concentrations of NAA used did not affect the frequency of embryogenesis or the number of somatic embryos produced. Similarly, in seedling segments, the

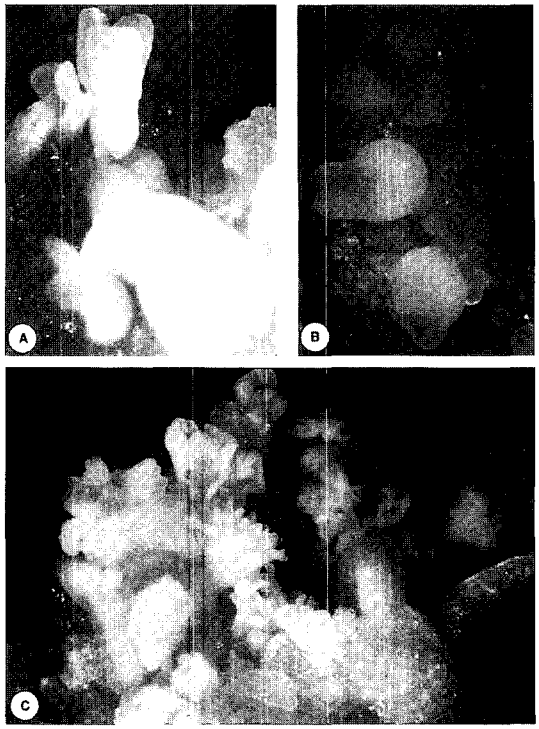

Fig. 5. Effect of a combination of 2,4-D and NAA on somatic embryo induction with cotyledonary (A, B) and zygotic embryo (C) explants. A. SEs induced on 10 μM 2,4-D and 5 μM NAA containing medium (13.6x). B. SEs induced on 15 μM 2,4-D and 15 μM NAA containing medium, showing small and globular stage SEs (23.8x). C. SEs with multiple cotyledons were observed on 5 μM 2,4-D and 15 μM NAA containing medium (6.8x).

optimal concentration of 2,4-D was 5 μM, giving a frequency of 27%. The frequency was reduced from 23 to 14% as the NAA levels increased from 5 to 15 μM. In addition, 98.5% of the total number of somatic embryos induced in seedling explants were formed on cotyledonary segments whereas shoot and root segments only formed 0.6% and 0.9% of the total somatic embryos, respectively.

For zygotic embryo-derived explants, the frequency of somatic embryo induction and number of somatic embryos were affected significantly by the concentration of 2,4-D in the media but not by the concentration of NAA. The optimal treatment was 5 μM 2,4-D which induced the formation of somatic embryos on 11% of the explants (Fig. 5C). In shoot explants, the highest frequency of somatic embryogenesis (62%) was recorded on explants

Table 3. Main effect of 2,4-D and NAA on somatic embryogenesis in cotyledonaray, seedling segments, zygotic embryo, and shoot (obtained from somatic embryos) explants of American ginseng after 8 weeks of induction.

Treatment (μM)		Frequency (%)				Number of Somatic Embryos Per Plate			
		Cotyledon	Seedling segments[z]	Zygotic embryo	Shoot[y]	Cotyledon	Seedling segments	Zygotic embryo	Shoot
2,4-D	5	58.2±4.0	27.7±3.0	11.7±3.1	62.2±3.4	7.8±0.6	6.4±0.1	0.5±2.0	15.5±0.9
	10	38.6±4.2	17.4±3.7	5.0±2.2	36.4±3.1	5.0±0.6	6.3±0.1	0.1±0.0	4.0±0.5
	15	27.8±3.2	10.6±3.0	33.6±1.7	24.4±2.5	2.8±0.5	6.2±0.1	0.1±0.0	2.2±0.4
	Trend[x]	L*	L*	L*	L*	L*			L*
NAA	5	41.2±4.5	22.8±3.9	8.3±2.8	39.0±3.6	3.7±0.5	6.4±0.1	0.3±0.1	4.9±0.6
	10	43.9±4.3	18.9±3.6	6.7±2.7	45.0±3.4	5.4±0.6	6.4±0.1	0.2±0.1	6.4±0.6
	15	39.2±3.8	14.2±2.9	5.4±2.0	38.9±3.3	5.5±0.6	6.0±0.1	0.2±0.1	5.4±0.6
	Trend		L*						
2,4-D				NS		NS	NS	NS	
NAA		NS	NS	NS	NS	NS	NS	NS	NS
2,4-D x NAA		NS	NS	NS	NS	NS	NS	NS	NS

[z]All segments of seedling.
[y]The concentrations of 2,4-D used for shoot explant were 2, 5, and 8 μM.
[x]Significant (*) at p=0.05, L-linear, Q-quadratic.
NS, *Nonsignificant or significant at p=0.05, respectively.

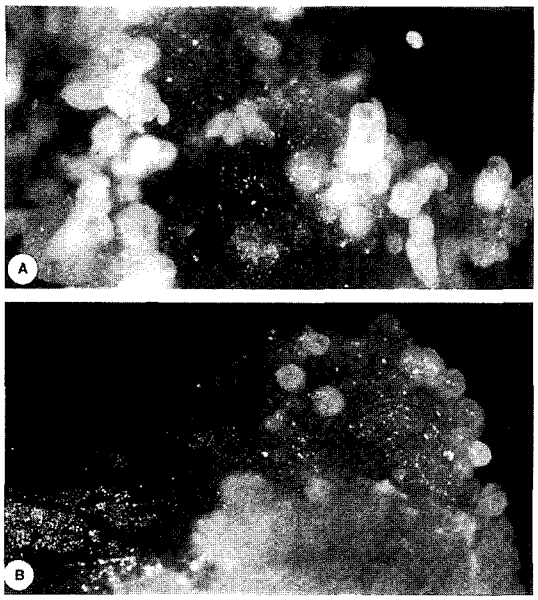

Fig. 6. Effect of a combination of 2,4-D and NAA on somatic embryo (SE) induction with shoot explants after 7 weeks of induction. A. SEs produced on 5 μM 2,4-D and 10 μM NAA containing medium (10.2x). B. SEs produced on 8 μM 2,4-D and 10 μM NAA containing medium, staying in early developmental stage (10.2x)

cultured on 2 μM 2,4-D supplemented medium whereas explants on 5 and 8 μM 2,4-D containing medium gave a frequency of 36% and 24%, respectively (Table 3, Fig. 6). The three concentrations of NAA used did not affect the response significantly.

(5) Light and scanning electron microscopy

Light microscopic observations confirmed that the observed regenerants were somatic embryos. Well-organized globular (Fig.7A), early cotyledonary (Fig. 7B) and cotyledonary-staged (Fig. 7C) somatic embryos were observed. The meristematic region was characterized by small cells with dense cytoplasm and highly stained nuclei, and the embryos were loosely attached to the explant and had no vascular connection with the maternal vasculature. The early stages of somatic embryogenesis were also observed by SEM (Fig.8 A, B). The morphologically mature cotyledonary stage somatic embryos resembled their mature zygotic embryo counterparts, but these were relatively smaller in size (10~60%). Both somatic (Fig. 8D) and zygotic embryos (Fig. 8E) had smooth cotyledonary surfaces. Some poorly developed, short, fused or multiple cotyledonary somatic embryos were also observed (Fig. 8C).

3. Effect of GA_3 and BAP on plantlet conversion and growth

(1) First generation SEs

Somatic embryos produced from stratified seed derived seedling explants were used in this experiment. After 4 weeks of culture on BM supplemented with 1.45 μM

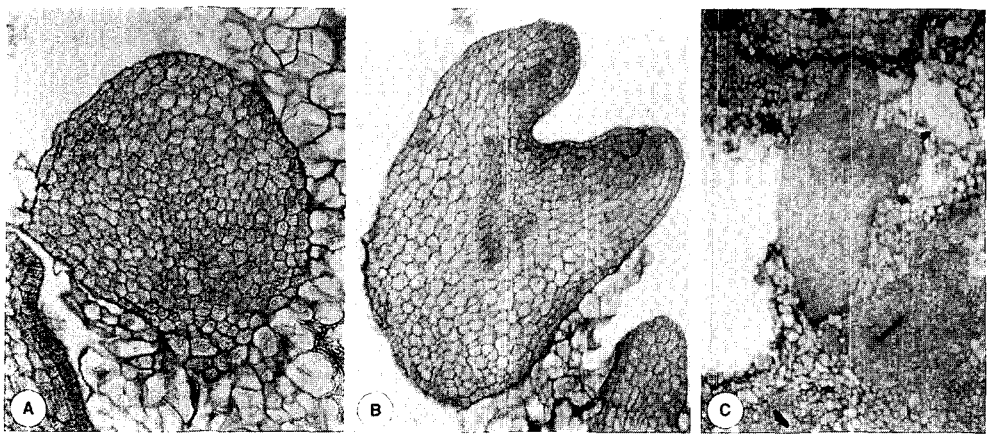

Fig. 7. Light microscopic observations of somatic embryogenesis in ginseng. A. Longitudinal section of cotyledonary explant after 8 weeks of induction on 2,4-D and NAA containing medium. Note: globular somatic embryos. B. Longitudinal section of cotyledonary explant after 8 weeks of culture on 2,4-D and NAA supplemented medium. Note: early cotyledonary stage embryo. C. Longitudinal section of cotyledonary explant after 8 weeks of induction on 2,4-D and NAA containing medium. Note: late cotyledonary stage somatic embryo.

GA_3, about 85% SEs were converted into plantlets which had well developed shoots and roots. However, on medium containing both GA_3 (1.45 μM) and BAP (2.2 μM) 58 % of the SEs converted into plantlets, and 25% of the SEs developed shoots only (Table 4).

In another experiment, SEs produced from green seed-derived seedling explants (with various pre-treatments) were used. The concentration of GA_3 (1 or 3 μM) as pre-treatment did not affect the conversion rate (Table 5). However, SEs produced from light-grown seedling explants gave higher conversion rate (47%) compared to those produced from dark-grown seedlings (31%).

(2) Second generation SEs

The highest conversion to plantlets occurred with SEs on 1.45 μM GA_3 containing medium (89%) after 4 weeks of culture (Table 6). The conversion rate dropped to 44% when 2.2 μM BAP was added to the medium, and 56% SEs converted to plantlets when 2.9 μM GA_3 and 4.4 μM BAP were included in the medium. Only 16% SEs developed into plantlets, while the most SEs (76%) remained small and did not develop further on BM containing no growth regulators.

In vitro flowering was observed in plantlets converted from secondary generation of SEs grown on GA_3 (1.45 or 2.9 μM) and BAP (2.2 or 4.4 μM) enriched half strength BM after 10 weeks of culture. On only 1.45 μM GA_3, containing medium, about 10% of the plantlets formed flowers. With plantlets grown on 1/2 BM enriched with other combinations of GA_3 and BAP, *in vitro* flowering was observed occasionally. Compared to the flowers on the greenhouse-grown 3-year-old plants, flowers of SE-derived plantlets were about 50% smaller and had significantly longer filaments. Single flowers were observed most of the time, although inflorescences with up to 12 flowers were also observed (Fig. 9 and 10). Flowers had 5 sepals and 5 petals (both were light-green in colour), and 5 yellow anthers (Fig. 11A). However, flowers with less or more than 5 anthers were also observed (Fig. 11B).

(3) Plantlet transplantation

Four-week-old plantlets grown on GA_3 containing medium were cultured on BM for 6 weeks (Fig. 12A) and then transferred to Jiffy 7 pellets and Promix-BX, and kept in a growth chamber (Fig. 12B). The measurement of 4 month-old plantlets showed that, both shoot and root height were about 19±2.7 mm; shoot number was 2.8±0.3; root diameter 2.9±0.3 mm; and leaflet number per shoot was 1.8±0.2. Figure 13 shows a 3 month-old plantlet with well developed shoots and tap roots.

The upper parts of the plantlets died back after 4-5 months in the growth chamber, but the roots were alive. After 3 months of storage at 3°C, a few roots were still alive and the bud started to emerge in the cold, and this

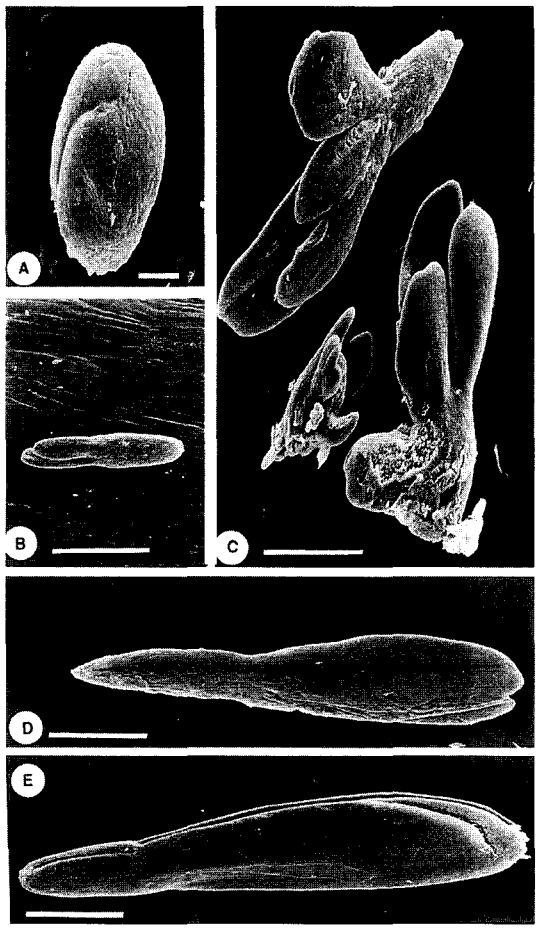

Fig. 8. SEM observations of somatic embryogenesis after 8 weeks of induction on 2,4-D supplemented medium (A-D) and a zygotic embryo (E). Bar=0.1 mm in A and 1.0 mm in B-E. A. An early heart shaped somatic embryo. B. An early cotyledonary stage embryo. C. A few abnormal somatic embryos with multiple cotyledons. D. A mature somatic embryo with two cotyledons. Note: smooth surface. E. A mature zygotic embryo with two cotyledons and a smooth surface.

process was similar to what occurred in greenhouse-grown seedlings.

Discussion

Somatic embryogenesis was induced by supplementing the culture medium with any of the three auxins and on all types of explants of American ginseng. The somatic embryos were observed primarily after 4 weeks of exposure to exogenous auxin. Explants generated from green seeds exhibited similar or higher embryogenic frequency than those by explants derived from stratified seeds.

The key role of exogenous auxin(s) in controlling somatic embryogenesis was clearly demonstrated by this study. Explants required a minimum 4 weeks of exposure time to 2,4-D for induction of high frequency and quality somatic embryos. Furthermore, the process of embryogenesis was mediated by both auxin type and concentration in the induction medium and the tissue response was dependent on explant types. Overall, the auxins 2,4-D and NAA were highly effective for somatic embryo induction, although their optimal concentrations were different, while dicamba induced the formation of significantly fewer embryos.

In the present study, in general, when equivalent concentrations of 2,4-D and dicamba were compared, the former was markedly more effective, both for the frequency of SE induction and number of SEs produced.

Table 4. Effect of GA_3 and BAP on rates of plantlet (conversion), shoot and other stages of American ginseng SEs after 4 weeks of culture[z].

Treatment			Rate (%)		
Basal media	GA_3 (μM)	BAP (μM)	Plantlet	Shoot	Other[y]
BM	1.45	0	84.7±2.7	2.7±1.1	12.6±2.6
BM	1.45	2.2	58.2±3.9	24.8±3.2	17.0±3.1

[z]First generation SEs which were derived from stratified seeds were used.
[y] Those were: 1) small SEs, did not develop further; 2) roots developed, but no shoots occurred; 3) green structure, but no shoot or roots could be identified.

Table 5. Effect of pretreatment for seedling growth on rates of plantlet (conversion), shoot and other SEs in American ginseng after 6 weeks of culture[z].

Pre-Treatment	Rate (%)		
	Plantlet	Shoot	Other[y]
Light	47.5±4.9	25.2±5.4	27.3±4.8
Dark	31.4±4.9	23.1±3.8	45.5±4.9
GA_3 (μM)			
1	40.8±4.5	23.0±4.2	36.2±4.5
3	39.0±5.5	25.2±5.2	35.8±5.4

[z]First generation SEs which were derived from green seeds were used. Culture medium for plantlet conversion consisted of BM and 1.45 μM GA_3.
[y]Those were: 1) small SEs, did not develop further; 2) roots developed, but no shoots occurred; 3) green structure, but no shoot or roots could be identified.

Table 6. Effect of GA_3 and BAP on rates of plantlet (conversion), shoot and other SEs in American ginseng (data collected after 4 weeks of culture)[Z].

Treatment			Rate		
Basal Medium	GA_3(μM)	BAP (μM)	Plantlet	Shoot	Other
MS	0	0	16.7±5.0	7.1±3.9	76.3±4.8
MS	1.45	0	88.8±4.3	2.3±1.6	9.1±2.6
MS	1.45	2.2	44.3±7.2	18.2±4.4	37.2±5.4
MS	2.9	4.4	56.8±6.0	13.6±3.9	29.4±4.2

[Z]Second generation SEs were used.

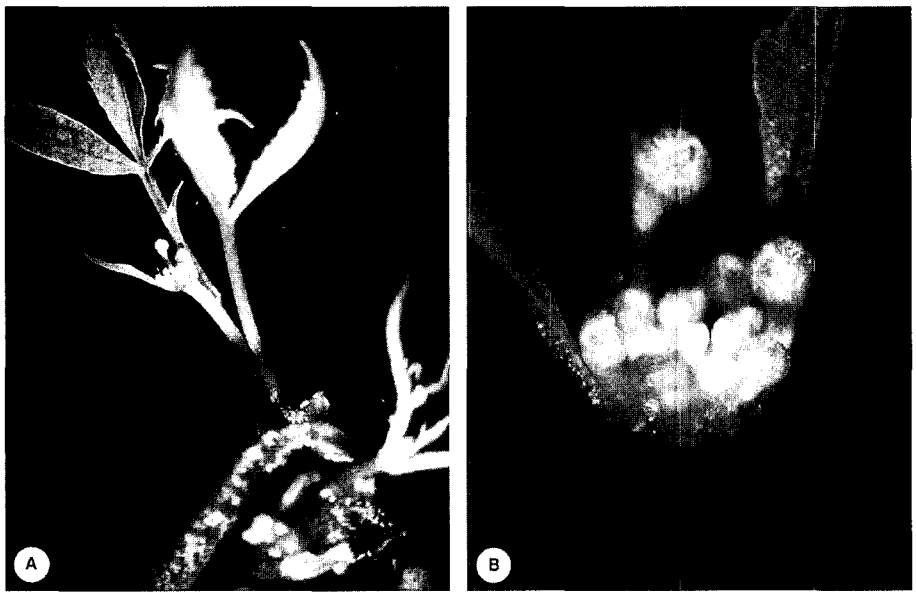

Fig. 9. An inflorescence developed from a somatic embryo-derived plantlet. A. About 12 flowers in an inflorescence (4.3x). B. Close up of A (17x).

Higher concentration (15 μM) of NAA was more comparable to lower concentration (5 μM) of 2,4-D with respect to the responses observed. In other plant species, different types of auxin are also not equal quantitatively or qualitatively in their action, and the effectiveness of an auxin is species and tissue specific (Gleddie *et al.*, 1983; Meijer and Brown, 1987; Hazra *et al.*, 1989; Jia and Chua, 1992).

Auxin concentration strongly affected the frequency and number of SE induced. The results indicated that the frequency and number of SE induction increased as the concentration of NAA increased from 5 to 15 μM in all three types of explants. With 2,4-D and dicamba, the highest frequency and number of SE were induced with 5 μM. Baker and Wetzstein (1994) reported somatic embryogenesis in peanut with 2,4-D or NAA, and showed that as auxin level increased, frequency of embryogenesis decreased. Reduction in embryogenic potential by high 2,4-D levels has also been reported for papaya (Fitch and Manshadt, 1990). On the other hand, frequency of embryogenesis in creeping bentgrass was increased from 57% to 71% when the concentration of dicamba increased from 5 μM to 10 μM (Zhong *et al.*, 1991).

The influence of auxin type on embryo morphology has been reported in many plants (Barwale *et al.*, 1986; Hartweck *et al.*, 1988; Tabei *et al.*, 1990). In the present study, some SEs obtained with NAA were of good quality, large, cotyledonary stage, and separate from each other, but others were often fused together and to the parental explant tissue. Good quality SEs were also induced with 2,4-D and dicamba, particularly with 5 μM. At higher concentrations, the SEs remained small

Fig. 10. An inflorescence observed in a somatic embryo-derived plantlet having 8 flowers (4.3x).

and at the globular stage. In addition, cup shaped SEs were often observed with 15 μM dicamba.

In the case of the combination of 2,4-D and NAA, the main effects of 2,4-D and NAA have been considered since no interaction between 2,4-D and NAA was found. Similar to when 2,4-D was used as a single growth regulator, higher frequency of SE induction and number of SE induced were associated with lower concentrations of 2,4-D in the presence of NAA. In the case of NAA, concentrations tested did not show a significant effect on embryogenesis in the presence of 2,4-D except with seedling segment explants in which lower concentration of NAA gave a higher frequency. Both the frequency and number of SEs increased as the concentrations of NAA increased when NAA was used as the only growth regulator. This result indicates that 2,4-D exhibited a much stronger morphogenic response than NAA in the ginseng somatic embryogenesis process.

Exposure time to 2,4-D significantly influenced the frequency and SE production of American ginseng in this study. Generally, exposure time of 4 or 6 weeks to 2,4-D (5 μM) was optimal to promote high embryogenic potential. The influence of exposure time in 2,4-D containing medium on somatic embryogenesis has also been significant in other crops (Cook and Brown, 1995; Marsolais *et al.*, 1991). Finstad *et al.*, (1993) showed that there was a consistent minimum requirement of expo-

Fig. 11. Flowers of somatic embryo-derived plantlets. A. Flower having 5 sepals, petals and anthers (6.4x). B. Flower having 5 petals and 8 anthers (6.4x).

Fig. 12. Plantlets developed from somatic embryos. A. Plantlets (6 weeks old) grown on BM (2.0x). B. Plantlet (3.5 month old) with two stems (2.0x).

Fig. 13. Three-month-old plantlet developed from a somatic embryo, showing three well developed shoots, a tap root and lateral roots (2.6x).

sure to growth regulators for embryogenesis to occur in alfalfa petiole culture. The requirement for 2,4-D for initiation of SEs in American ginseng has been reported by Li and Guo (1990), whereas SE production can occur on basal medium without growth regulators in Oriental ginseng (Choi *et al.*, 1996; Lee *et al.*, 1990). The present study indicated that somatic embryogenesis of American ginseng can occur on basal medium but it is necessary for explants to have 4-6 weeks of exposure to 2,4-D supplemented medium as the inductive stimuli and the result was comparable to, or even better than, those obtained from explants which remained on 2,4-D containing medium for the whole period of induction. In carrot somatic embryogenesis, auxin (2,4-D) was required as the inductive stimuli but auxin removal was necessary for embryo development (Li, 1992). The occurrence of SE with shoot explants on basal medium may be a result of the carry-over effect of the explant since they were derived from germinating SEs.

In the present study with first generation SEs, about 85% of the SEs derived from stratified seeds converted into plantlets which had both shoots and roots after 4 weeks of culture on 1.45 μM GA_3 containing medium. On the contrary, 58% of the SEs converted into plantlets and 25 % of the SEs developed shoots only when 2.2 μM BAP was also in the medium. It would therefore appear that the presence of BAP in the culture medium inhibited root formation in some of the SEs (Gill *et al.*, 1995; Van Schaik *et al.*, 1996). Similarly, with second generation of SEs, plantlet conversion was 88% with SEs on medium supplemented with 1.45 μM GA_3 and conversion rate reduced to half when BAP was also in the medium, and 56% of the SEs converted into plantlets on 2.9 μM GA_3 and 4.4 μM BAP containing medium. Tirajoh and Punja (1995) reported that in the presence of 2.9 μM GA_3 and 4.4 μM BAP, SEs which were derived from root, leaf, and epicotyl explants of American ginseng did not develop roots after 1.5 months of culture. The different results obtained with 2.9 μM GA_3 and 4.4

μM BAP containing medium could be the result of different explant source, and the influence of the culture history (Ranch *et al.*, 1985). In alfalfa, SEs initiated on medium containing zeatin-riboside and 2,4-D usually produced both shoot and roots, while SEs cultured on BA and 2,4-D medium usually had retarded primordial roots (Kao and Michayluk, 1981).

In this study a low conversion rate was obtained with BM after 4 weeks of culture and addition of 1.45 μM GA_3 to the medium significantly improved conversion rate. Jia and Chua (1992) reported that both cytokinin and auxin were required for SE maturation and germination in *Pharbitis nil*, and abnormally swollen structures instead of normal plantlets were obtained from SEs which were directly transferred to basal medium without growth regulators.

The ginseng plant usually has one solitary shoot. However, many of the plantlets developed from SEs had more than one shoots, often two or three. This could be related to the environment of in vitro culture, and the effect of growth regulators in the growth medium. Seedlings germinated from ZEs of Oriental ginseng also developed 2~3 shoots on 5 μM GA_3 containing medium (Lee *et al.*, 1991).

Plantlets derived from second generation SEs formed flowers on BM supplemented with GA_3 alone or combined with BAP after 10 weeks of culture. Usually, American ginseng plants need a 2~3 year juvenile period to reach the flowering age in the field (Proctor and Bailey, 1987). As demonstrated by our results, in vitro culture shortened the flowering period to a few months. In vitro flowering has also been observed in Oriental ginseng with SEs (Chang and Hsing, 1980; Shoyama *et al.*, 1988; Lee *et al.*, 1995), and ZEs (Lee *et al.*, 1991): these studies indicated that BA but not GA_3 was required for in vitro flower induction. However, in the present study GA_3 alone produced more flowering than when in combination with BA.

In the course of this study we have developed and optimized a protocol for the micropropagation of American ginseng via somatic embryogenesis. Both the type and concentration of auxin in the culture medium were essential for the efficient regeneration of ginseng. Somatic embryogenesis was induced on cotyledonary, zygotic embryo and shoot-derived explants. Somatic embryos germinated and were grown into complete plants in the greenhouse. The regeneration system developed in these studies may provide the basis for the further improvement of the American ginseng crop through the application of molecular techniques.

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