

## Effects of Date and Growth Regulators on the Culture of Immature Zygotic Embryos of North American Ginseng

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**Abstract :** As the zygotic embryo of North American ginseng (*Panax quinquefolius* L.) matured during stratification over 203 days it grew from 0.75 to 5.2 mm. Embryo excision and culturing on media containing different concentrations of two growth regulators, gibberellic acid (GA<sub>3</sub>, 1 to 10 μM) and benzyladenine (BA, 1 to 5 μM), during stratification, showed that shoot and root number and the shoot, root and cotyledon length increased with increased stratification time. Gibberellic acid was the more effective growth regulator for increasing shoot and root number and shoot, root and cotyledon lengths. Immature embryos (stratified for up to 63 days) needed growth regulators for further development. Cultures on GA<sub>3</sub> at the last culture date (stratified for 203 days) when embryos were mature, produced multiple shoots but there was no effect of GA<sub>3</sub> concentration. Benzyladenine inhibited shoot and root growth regardless of embryo stratification. Growth regulators had little effect on cotyledon length of mature embryos. Embryos cultured on GA<sub>3</sub> combined with BA were green on all culture dates whereas greening in the control and BA treatments increased with culture date. The BA treatments induced 100% swelling of the embryos on the final culture date while in the control and GA<sub>3</sub> treatments there was no swelling. There was little or no curling in the control and BA treatments and a linear decrease in curling with culture date in the GA<sub>3</sub> and GA<sub>3</sub> + BA treatments.

**Key words :** *Panax quinquefolius*, plant regeneration, propagation, seed dormancy

### INTRODUCTION

North American ginseng (*Panax quinquefolius* L.) is a perennial herbaceous plant grown for its highly-valued, fleshy root.<sup>1,2)</sup> Ginseng is propagated by seed which requires a long (18 to 24 months), expensive and disease-prone stratification period.<sup>3-5)</sup> An alternative propagation method is desirable. Propagation of North American ginseng by tissue culture techniques has been disappointing, producing limited results from callus, leaves, petioles, hypocotyls, stems roots and zygotic embryos.<sup>6-10)</sup> Using the zygotic embryo as the explant, particularly from immature seeds, has produced encouraging results for rapid somatic embryogenesis.<sup>11)</sup> Recently, Zhou and Brown<sup>12)</sup>, using excised mature zygotic embryos for somatic embryogenesis, optimized a micropropagation procedure for North American ginseng resulting in high regeneration efficiency and successful transplanting into the field. Mass vegetative propagation using the zygotic

embryo, and studies on seed stratification with the potential for spring seeding of North American ginseng<sup>13, 14)</sup>, necessitate an understanding of the growth and development of the excised immature zygotic embryo of ginseng in culture.

The media and growth regulator requirements for zygotic embryo culturing differ between species and with physiological maturity of the embryo.<sup>15)</sup> Many mature embryos can grow normally on media without the addition of growth regulators.<sup>15)</sup> However, if growth regulators are required, those most commonly used to induce immature embryos to germinate *in vitro* are gibberellic acid (GA<sub>3</sub>), 6-benzylaminopurine (BA), kinetin, 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D).<sup>16-20)</sup> Li and Guo<sup>8)</sup>, using mature embryos and media supplemented with 2,4-D and GA<sub>3</sub>, obtained, through somatic embryogenesis, callus, embryoids and eventually plantlets of North American ginseng through somatic embryogenesis. The objective of this study was to determine the effects of GA<sub>3</sub> and BA on the germination and subsequent development of immature zygotic embryos of North American ginseng during stratification.

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## MATERIALS AND METHODS

The seeds for this investigation were harvested from a producer's 4-year-old ginseng planting at the end of August, graded into 4 sizes and held at  $15 \pm 0.3^\circ\text{C}$ . The largest seeds,  $> 5.9\text{mm}$  diameter, were mixed with mortar sand (3 sand: 1 seed, v/v) and stored in 2-L plastic containers at  $15 \pm 0.3^\circ\text{C}$  from 24 September until 24 January and  $3 \pm 0.2^\circ\text{C}$  from 25 January until 9 May. The moisture content of seed/sand mixture was maintained at about 15% moisture.

Embryo culturing was done at the end of each month from September until May, giving 8 culture dates except that on culture date 4 (December) no culturing was done but the seeds were moved from 15 to  $3^\circ\text{C}$ . At each culture date, except 4, the seeds were removed from storage, sifted from the sand and soaked in freshly made 2.5% sodium hydroxide for 20 minutes to facilitate coat (endocarp) removal.<sup>21)</sup>

Before excision of the embryos from the endosperm, the intact embryo and endosperm were surface sterilised in a 40% Javex (Colgate Palmolive, Toronto, Ontario; 5.25% sodium hypochlorite) solution with 2 drops of Tween 20 under a laminar flow hood for 20 minutes. After sterilisation, the seeds were rinsed for 2 minutes in sterile deionized water and the process was repeated 5 times. The embryos were excised under a dissecting microscope using microdissecting forceps and scalpel and plated aseptically onto the media. Each treatment was replicated 6 times with 6 embryos per plate. Each culture dish was double wrapped with Parafilm® and placed in a growth chamber at  $24 \pm 0.5^\circ\text{C}$  with a 16-h photoperiod ( $30$  to  $35 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by cool-white fluorescent tubes (Philips Canada, Scarborough, Ontario).

The experiment consisted of 17 treatments containing different concentrations of  $\text{GA}_3$  and BA: treatments 1 to 8 (0, 1, 2, 3, 4, 5, 6 and  $10 \mu\text{M}$   $\text{GA}_3$ ); treatments 9 to 11 (1, 3 and  $5 \mu\text{M}$  BA) and treatments 12 to 17 (combinations of 1, 3 and  $5 \mu\text{M}$   $\text{GA}_3$  and 1 or  $3 \mu\text{M}$  BA). Each culture dish contained 25 ml of the culture medium. The culture medium contained standard salt-mixture of MS formulation<sup>22)</sup>, B5 vitamins<sup>23)</sup>, 3% sucrose and 6.7 mM of 2-(N-morpholino)-ethane-sulfonic acid (MES), adjusted to a pH of 5.7. Then 150 ml of medium was poured into 17 Erlenmeyer flasks (1 flask/treatment) and 0.25% Gellan gum (Schweizerhall Inc., South Plainfield, N.J.) was added to each flask prior to autoclaving at  $1.19 \text{ kg}\cdot\text{cm}^{-2}$  for 20 minutes at  $121^\circ\text{C}$ . When the media had cooled to about  $55^\circ\text{C}$  the growth regulators were added following sterilisation

with a Millex-GV 0.22  $\mu\text{m}$  filter unit (Millipore) in a laminar flow hood.

The embryos were incubated in the growth chamber for 6 weeks at which time embryo germination, and contamination, were recorded. The growth parameters measured for germinated embryos were: number of shoots (SN) and roots (RN), length of each shoot (SL), root (RL) and cotyledon (CL), whether the germinated embryo was notably swollen or not, the color of the germinated embryo (4 colours were seen: green, yellow, white and brown and given rank numbers of 4, 3, 2 and 1 respectively), and the amount of curling of the germinated embryo which was done on a 6-point grading system (0-no curling, 1-slightly curled, 2-beginning to coil, 3-coiled but with all botanical parts still distinguishable, 4-coiled and botanical parts not all distinguishable and 5-tightly coiled, indistinguishable ball).

All the growth parameter data, except for swelling, were normalised using either log, square root or power transformation. The data were then analysed using the General Linear Models (GLM) program of the SAS statistical package (SAS Institute, Cary, NC). Data for seed contamination was not included in the model since any embryo that was contaminated did not germinate. The germination and swelling results were subjected to the Logistic Linear Model (Logistic) program of the SAS statistical package (SAS Institute, Cary, NC) which takes into account non-normal data. The differences between the culture date means and the treatment germination and swelling means were computed using contrasts between the means. Linear and quadratic contrast were performed on the culture date means and treatment germination and swelling means. Contrasts between the different groups of growth regulator treatments were also performed. Stratification temperature effects were not determined since these were confounded with culture date. Stoltz and Snyder<sup>24)</sup> used a similar approach.

The difference between the culture date and treatment yield component means were analysed using the LSMEANS statement of the General Linear Models (GLM) program of the SAS statistical package (SAS Institute, Cary, N.C.). Linear and quadratic contrasts and contrasts between the different groups of growth regulator treatments were performed.

## RESULTS

### Embryo Germination and Contamination

Embryo germination, defined as ending with the start of elongation of the embryonic axis<sup>52)</sup>, occurred in all 17

treatments at each culture date. Any embryo that was not contaminated germinated. A negative correlation ( $r = -0.93$ ) was found between germination and contamination. Because of this correlation all data for seeds that were contaminated (about 30%) were removed from the data set. Once data for seeds with contamination were removed, the germination at each culture date and for each treatment was about 100% and there was no statistical significance between culture dates or treatments.

Often during embryo excision a suspensor was seen (Fig. 1). We believe that this is the first report of a suspensor in North American ginseng. The suspensor functions as the site of active uptake and transport of nutrients to the embryo and plays a regulatory role in zygotic embryogenesis<sup>26</sup>. Very young excised embryos do not develop further in culture if the suspensor is removed<sup>27</sup> unless GA is added to the medium<sup>25, 28</sup>. The role of the suspensor in ginseng embryo culture was beyond the scope of this study.

#### Effect of culture date and treatment on embryo growth and development

During stratification two embryo growth stages, one slow and the other faster, were identified (Fig. 2). Stage I was from September to December, the 15°C stratification period, when embryo length increased from 0.75 to 1.6

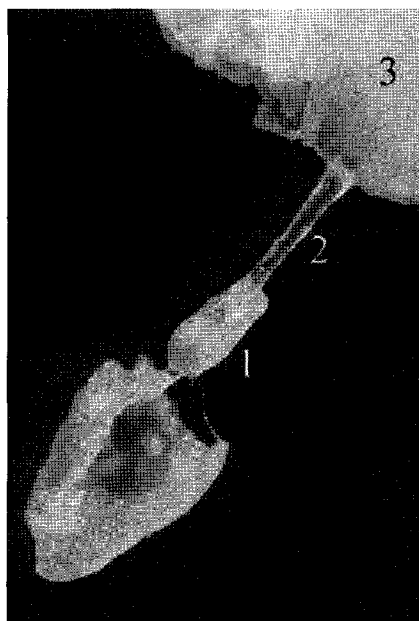


Fig. 1. The excised zygotic embryo (1) about 3 mm long, suspensor (2), and the seed endosperm (3), of a North American ginseng seed; magnification X6.

mm ( $0.008 \text{ mm day}^{-1}$ ). In stage II, embryo length increased from 1.6 to 5.2 mm ( $0.036 \text{ mm day}^{-1}$ ). As the embryo grew from 0.75 to 5.2 mm its inherent ability to develop into a complete plant increased.

Shoot and root number (Fig. 3) and length (Fig. 4) increased with culture date in all treatments. Shoot number increased from 0.13 on culture date 1 to 1.31 for date 8. Root number increased from 0.15 on culture date 1 to 1.03 for date 8. By culture date 8, a fully formed seedling with a root, two cotyledons and at least one shoot could be obtained.

Generally, the greatest growth responses were found with GA<sub>3</sub> followed by the combination of GA<sub>3</sub> and BA, and then BA alone. In regression analysis the amount of variation accounted for by the linear relationship between shoot and root number and culture date was high for the GA<sub>3</sub>/BA mixture and BA alone (range 76 to 91%) but low for GA<sub>3</sub> alone, 46% for shoots and 63% for roots. Shoot length responded to the growth regulator treatments much like shoot and root number (Fig. 4). Root length was similar for GA<sub>3</sub> and the control and greater than BA alone, or in combination with GA<sub>3</sub> after the warm period of stratification was complete (Fig. 4).

The effects of different concentrations of growth regulators were similar within each culture date. To illustrate this data from the final culture date are presented below. This date was chosen as it was the time when most complete seedlings were obtained. Three distinguishable groups were found (Figs 5-7) and they relate to the three growth regulator treatments. The GA<sub>3</sub> group for each variable resulted in the highest shoot and root numbers

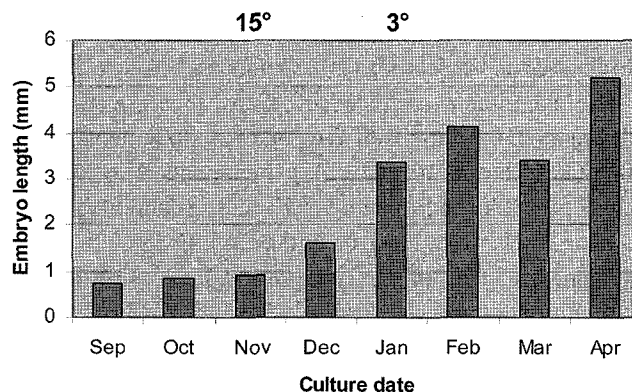


Fig. 2. Embryo length of North American ginseng seed on 8 culture dates. Culture date 1 was September 29 and April 29 was Culture date 8. Seeds were transferred from 15°C to 3°C on Culture date 4 (Dec. 24) but no embryo culturing was carried out. Embryo length on Culture date 1 was 0.75 mm.

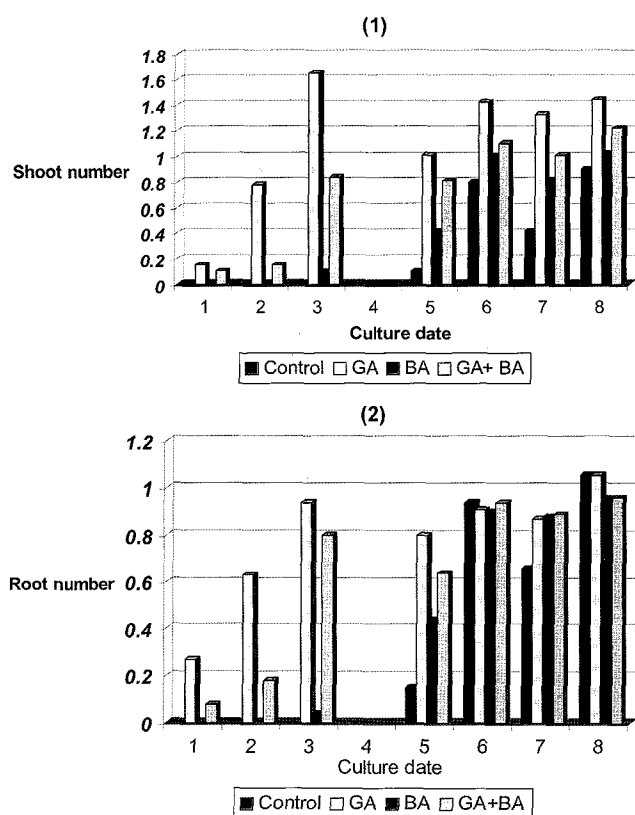


Fig. 3. Effect of culture date and three growth regulator treatments (see Figure legend) on shoot number and (2) root number of North American ginseng zygotic embryos cultured *in vitro*. Culture date 4 was when seeds were transferred from 15° to 3°C and no embryo culturing was carried out.

and the longest shoots, roots and cotyledons. Embryos on GA<sub>3</sub> produced the most shoots (1.47) and all GA<sub>3</sub> concentrations gave similar results (Fig. 5). Cultures without growth regulators produced the fewest shoots (0.89), those on BA formed 1.08 and those on GA<sub>3</sub> + BA formed 1.23 shoots. Similar results were found for shoot length (Fig. 6).

Root production was similar whether or not growth regulators were included in the culture media (Figs 5-7). Generally, one root per embryo was obtained and this would be necessary if these seedlings were to be transferred to other media e.g. soil, for further studies. The GA<sub>3</sub> treatments gave the longest roots and the GA<sub>3</sub> + BA mixture had no effect or reduced it, particularly at high concentrations.

#### Qualitative assessment of plantlet color, curling and swelling

The embryos cultured on GA<sub>3</sub> and the GA<sub>3</sub> + BA mix-

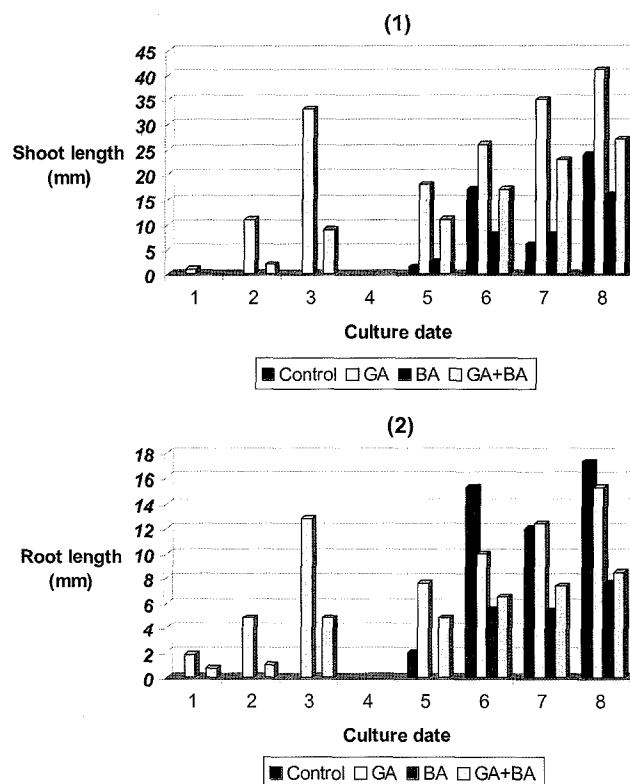


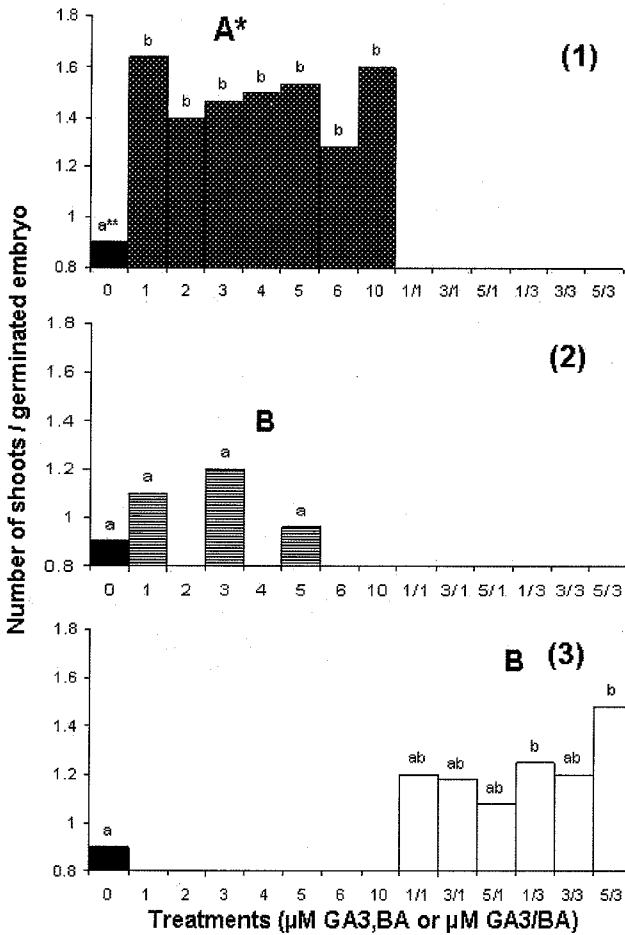
Fig. 4. Effect of culture date and three growth regulator treatments (see Figure legend) on shoot length and (2) root length of North American ginseng zygotic embryos cultured *in vitro*. Culture date 4 was when seeds were transferred from 15° to 3°C and no embryo culturing was carried out.

ture were green on all culture dates whereas greening in the control and BA increased linearly with culture date (data not shown). On the final culture date embryos on only the lowest concentration (1 μM) of GA<sub>3</sub> and BA were greener. Embryo and plantlet curling decreased linearly with culture date in the GA<sub>3</sub> and the GA<sub>3</sub> + BA combination treatments and there was little or no curling in the control and BA treatments. By the last culture date, the GA<sub>3</sub> treatments increased curling, particularly at the higher concentrations, BA had no effect and some of the GA<sub>3</sub> + BA combinations increased curling. Swelling showed no pattern with culture date. On the final culture date there was no swelling in the control and GA<sub>3</sub> treatments and 100% swelling in the BA treatments.

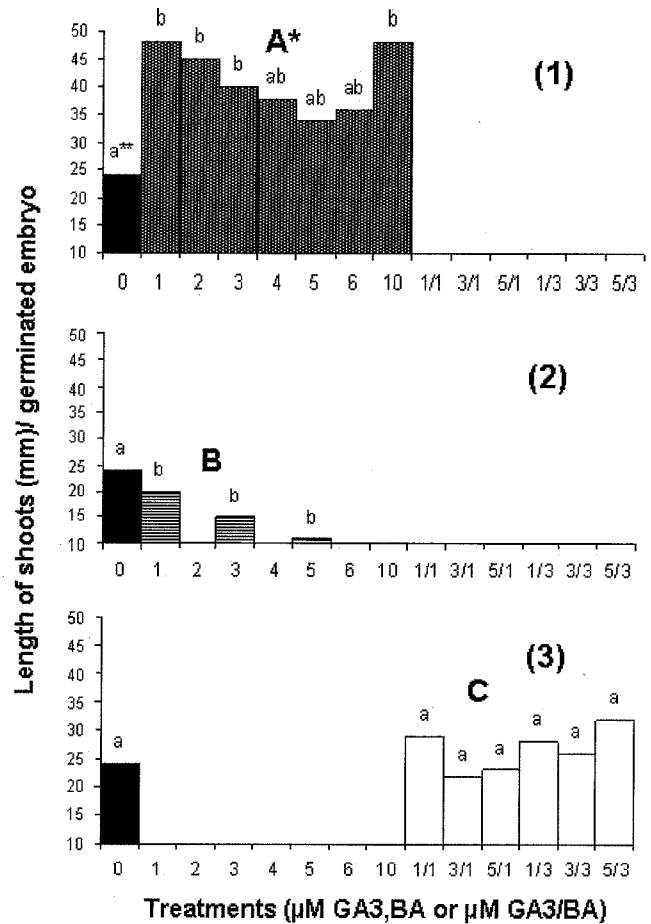
## DISCUSSION

### Germination and contamination

There are a number of possible reasons for the high rate



**Fig. 5.** Comparison of shoot number per embryo of North American ginseng zygotic embryos culture *in vitro* on the last culture date (8) for each group of treatments; control and GA<sub>3</sub>, (2) control and BA and (3) control and GA + BA. \*Groups with different letters are significant. \*\*Bars with different letters within groups are significant.



**Fig. 6.** Comparison of shoot length per embryo of North American ginseng embryos cultured *in vitro* on the last culture date (8) for each group of treatments; (1) control and GA<sub>3</sub>, (2) control and BA and (3) control and GA + BA. \*Groups with different letters are significant. \*\*Bars with different letters within groups are significant.

of embryo contamination. Tianyu *et al.*,<sup>29)</sup> and Ziezold *et al.*,<sup>5)</sup> analysed *Panax quinquefolium* outer and inner seed coats and endosperm for the presence of fungi. Different species of *Cylindrocarpon*, *Fusarium*, *Alternaria*, *Trichoderma*, *Drechslera*, *Penicillium*, *Aspergillus* and *Trichothecium* were found on the outside of the seed coat. On the inside of the seed coat Tianyu *et al.*,<sup>29)</sup> found different species of *Fusarium*, *Cylindrocarpon*, *Alternaria* and *Penicillium*. The fungi inside the endosperm were *Cylindrocarpon*, *Trichoderma*, *Fusarium* and *Alternaria*. Tianyu and Weiqun<sup>30)</sup> found that *Cylindrocarpon* is present during flowering and subsequent development of the seed on the plant. During embryo culturing, in the present study, the seed coat was removed and the seed

endosperm was surface sterilized. The period of surface sterilization may not have been long enough and/or the concentration of the sterilant, too weak to kill the fungi present on the inside of the seed coat which were in contact with the endosperm. Only the outside of the endosperm was sterilized so any fungi inside the endosperm, in contact with the embryo, were not killed. The susceptibility of *in vitro* cultures to pathogens may be another reason for contamination.

Growth regulators were not necessary for germination of the embryos since the embryos in all control (no growth regulators) cultures grew. Hu and Wang<sup>31)</sup> concluded that growth regulators were not required in embryo culture media for most species, except for very young

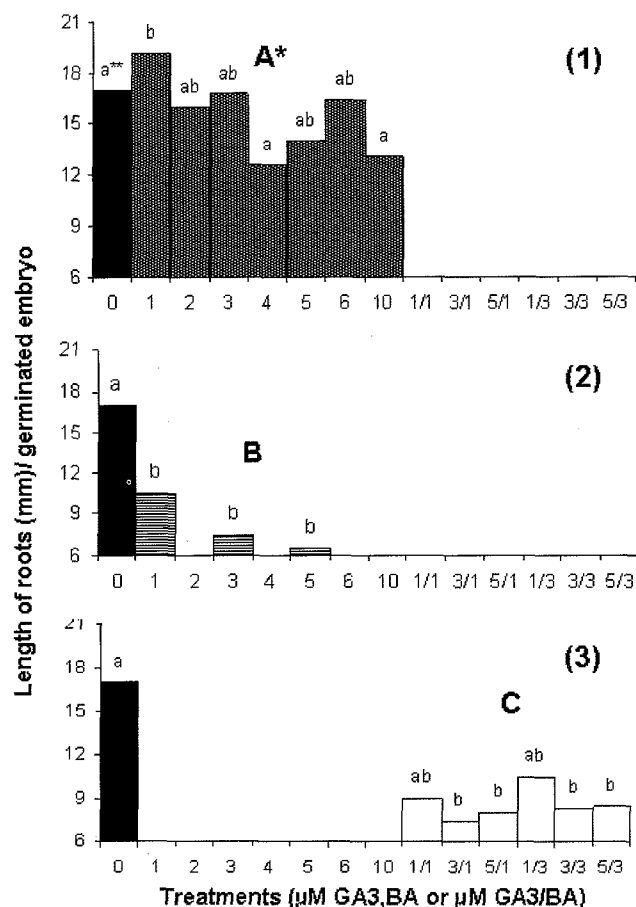


Fig. 7. Comparison of root length per embryo of North American ginseng embryos cultured *in vitro* on the last culture date (8) for each group of treatments; (1) control and GA<sub>3</sub>, (2) control and BA and (3) control and GA + BA. \*Groups with different letters are significant. \*\*Bars with different letters within groups are significant.

embryos and in clonal multiplication. In many species removal of the embryo from the endosperm and placement on nutrient supplemented culture media resulted in embryo germination<sup>27</sup>. Choi and Soh<sup>32</sup> found that germination of intact zygotic embryos of *Panax ginseng* occurred at all stages of development except for immature torpedo, when cultured on MS basal medium.

The extent to which the embryo develops into a mature plantlet varies from species to species and with the physiological maturity of the embryo<sup>27</sup>. GA<sub>3</sub> and BA affected the development of the germinating embryo into a plantlet, as shown by analysis of the growth parameters, shoot and root number and shoot, root and cotyledon length and embryo color, swelling and curling (discussion below).



Fig. 8. Multiple shooting of a North American ginseng zygotic embryo cultured *in vitro* on media containing 5  $\mu\text{M}$  GA<sub>3</sub>; magnification X2.

#### Effect of culture date and treatment on embryo growth and development

The two-stage embryo growth period of 8 months (September to April, Fig. 2) differs from the traditional three-stage 18 month period<sup>3</sup>. The differences are due to the controlled-temperature stratification<sup>4</sup> of 15EC followed by 3EC each for 4 months compared to outdoor stratification box temperatures of cold/warm/cold. The embryo growth rate at 15EC was 0.008mm day<sup>-1</sup> which is about 4 times greater than at the low temperatures (range 12EC to 2EC) of the standard outdoor stratification box. Growth in Stage II (3EC) of controlled temperature and Stage III outdoors were similar (0.036 mm day<sup>-1</sup> vs 0.03 mm day<sup>-1</sup>). The pattern of embryo development in ginseng was similar to that in *Arabidopsis thaliana* L. Heynh, a model plant species for fundamental research on growth and differentiation.

Kost *et al.*,<sup>18</sup> studied the regeneration ability of fertile plants of *Arabidopsis thaliana* by culturing the embryos at different stages of development. At all stages the *A. thaliana* embryos germinated, as found in this study with ginseng. However, fewer shoots were obtained from younger embryos.

The ginseng plant has a single solitary stem which exhibits strong apical dominance<sup>2</sup>. In the present *in vitro*

study, as in earlier work on somatic embryogenesis<sup>11)</sup>, multiple shooting occurred (Fig. 8). Morphological differentiation of cultured embryos are often described as somewhat abnormal<sup>18)</sup> and multiple shooting is a very common phenomenon<sup>18)</sup>. Kost *et al.*,<sup>18)</sup> suggested that multiple shooting was a consequence of the high developmental potential expressed by the apical region of dicotyledonous embryos, which is difficult to control *in vitro*. Sometimes in cultivation a small number (<10%) of older plants have 2 or rarely 3, stems<sup>2,33)</sup>. Multiple stemmed ginseng plants had a 50% greater root weight than those with solitary stems<sup>33)</sup>. Therefore, this finding of multiple shoots *in vitro* may have economic gains for growers due to higher yields.

In the present study, growth in the early stages of embryo development of the SL and RL were much slower compared to later dates (Fig. 4). Embryo survival, germination and plantlet growth *in vitro* depends on the medium used and the stage of embryo development<sup>34)</sup>. Generally, the younger embryo, the more complex the media required to facilitate embryo growth. The media used at each culture date in the present study did not vary in terms of nutrient salts and vitamin supplementation and concentration of growth regulators. Changes in the media components may be necessary to determine if greater SL, RL and CL's are achievable.

By culture date 8 the highest number of shoots and roots and the greatest lengths were produced from embryos cultured on media containing GA<sub>3</sub> (Figs 5-7). Under normal embryo development conditions, GA is secreted to the aleurone layer of the seed which responds by synthesizing and secreting α-amylase to the endosperm<sup>35)</sup>. The enzyme α-amylase begins the hydrolysis of starch reserves and the final products are obtained by the embryo from the transfer cells of the scutellum. *In vitro* culturing of embryos removes the aleurone layer and the endosperm (food reserve). The addition of GA<sub>3</sub> to the media substituted for the aleurone tissue as the source for GA<sub>3</sub> and the media replaced the endosperm.

#### Qualitative assessment of plantlet color, curling and swelling

In this trial, greening of embryos and plantlets was related to GA<sub>3</sub> and embryo maturity. Choi and Soh<sup>32)</sup> found that immature cotyledons of *Panax ginseng* were white and turned yellow-green after 4 weeks in culture while cotyledons in germinating embryos had both green and red pigments. In this study, embryo maturity played a major role in plantlet greening. As the embryo matured

the length of the shoots increased linearly with time. As plant leaves grow and mature the ability to photosynthesize increases (until the leaves are fully expanded). Presumably, the longer shoots had larger leaf expansions therefore greater areas for light interception. Additionally, the maturity and corresponding pigments may modulate plantlet growth as a result of light-induced photomorphogenic processes.

Growth regulators may also play a role in plant greening. In culture the media containing GA<sub>3</sub> resulted in the longest shoots and presumably largest leaf areas. A prominent physiological effect of gibberellins in developing plants, including ginseng, is elongation of plant parts<sup>36,37)</sup>. This growth promoting effect is caused mainly by cell elongation and partly by cell division<sup>38)</sup>. As explained above larger leaf areas have greater potential for light interception.

The BA effect on greening was most evident in the mature embryos since there was a linear increase in green color with culture date. Cytokinins promote rapid cell division and differentiation which promotes activities such as chlorophyll production<sup>37)</sup>. BA may have resulted in increased levels of chlorophyll in the mature embryos *in vitro*.

The large amount of embryo and plantlet swelling in GA<sub>3</sub> + BA mixtures was a BA effect. Sansberro *et al.*,<sup>39)</sup> found that on media containing cytokinins, some embryos grew normally with suppression of root growth and precocious leaf expansion. The response of tissue cultures to exogenous cytokinins is a complex function of several biochemical processes<sup>40)</sup>. In plant development cytokinins function by increasing the rate of chloroplast biogenesis and of synthesis of ribulose biphosphate carboxylase, delay senescence, stimulate nucleic acid synthesis and cell division<sup>41)</sup>. The excessive swelling seen in the present study was a direct result of the BA within the tissue culture media promoting rapid cell division within the explants. This abnormal swelling may have arrested further development of the plantlets<sup>32)</sup>. Cytokinins, and the plant growth regulators with cytokinin-like activity, are known to stimulate expansion, organization and adventitious shoot growth in cultured seeds and embryos<sup>42)</sup>.

In conclusion, Asian ginseng seed can be induced by a warm/cold treatment to germinate in 8 to 10 months after seed harvest. The *in vitro* work reported here shows, for the first time, that if freshly harvested North American ginseng seed is exposed to a warm/cold regime, embryo growth, germination, shoot growth and concurrent radicle and root elongation, take place. The growth regulator, gib-

berellic acid, has a stimulatory effect on these processes. A possible application of this work would be the selection of immature (unstratified) seeds in the field from breeding lines and the culture of their immature embryos, or parts of them, for somatic embryogenesis. Another application of these findings is the potential to implement spring seeding of North American ginseng seed. Traditionally North American ginseng seed has been exposed to an 18 month cold/warm/cold regime for embryo maturation and germination. Shortening this 18 month period to 8 months would facilitate spring seeding and reduce damping-off and root rot diseases that substantially reduce crop stand and performance.

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