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Rapid *in vitro* Germination of Zygotic Embryos *via* Endosperm Removal in *Eleutherococcus senticosus*

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Abstract

Eleutherococcus senticosus **(also called** ***Acanthopanax senticosus*****), belonging to Araliaceae family, has been used as an important medicinal woody plant. Mature seeds of** ***Eleutherococcus senticosus*** **have rudimentary (extremely immature) zygotic embryos and require a long-term stratification for about 18 months to induce germination. Here, through the methods of endosperm removal and other exogenous treatments, we investigated the factors for inducing rudimentary embryos by** ***in vitro*** **culture. Rudimentary zygotic embryos in seeds were at globular to heart-shaped stage at about 250 μm in length just after harvest of fruits. When the seeds without testa were cultured on 1/2 MS (Murashige and Skoog 1962) medium, they did not germinate regardless of medium and sucrose concentrations but the removal of endosperm tissue markedly stimulated the growth of rudimentary zygotic embryos. The embryo reached earlier maturation, once when the endosperm surrounding the rudimentary embryos was removed. Rudimentary zygotic embryos developed cotyledons within 3 weeks of culture after endosperm removal. However, post-mature zygotic embryos failed to germinate though they were morphologically normal, indicating another dormancy of embryos. GA_3 (2.0 mg/L) and/or charcoal (0.2%) treatment rapidly enhanced the germination of zygotic embryos. These results suggest that** ***E. senticosus*** **seeds have double dormancy; i. e. morphological rudimentary dormancy influenced by surrounding endosperm and physiological dormancy after post-maturation of zygotic embryos. Based on the above findings, we established the rapid germination of rudimentary zygotic embryos by** ***in vitro*** **culture of excised seeds with endosperm removal and GA_3 treatment.**

Key words: Zygotic embryo, Rudimentary embryo, *Eleutherococcus senticosus*, Endosperm removal, Morphological and physiological dormancy

Introduction

Eleutherococcus senticosus (also called *Acanthopanax senticosus*), belonging to Araliaceae family, is distributed throughout Northeast Asia. This plant has been used as an important medicinal woody plant, and its root bark contains important medicinal components which play an effective role for tonic, anti-rheumatic, prophylactic, and etc (Brekham 1960). *E. senticosus* seeds contain rudimentary zygotic embryos just after harvest of fruits. Zygotic embryos in seeds must undertake the development from the early heart-shaped to the cotyledonary stage before seed germination (Liu *et al.* 1998). Zhu and Wang (1992) investigated that the germination ratio from seed to seedling was 7.9% and took 2 years in the natural habitat due to strong dormancy. Through the treatment of stratification (15℃), it took 18 months for the dormancy breaking (Isoda and Shoji 1994). Park et al. (1997) demonstrated that the combination of low temperature (15℃) and gibberellic acid (GA_3) could effectively break the dormancy of seed and germination, and it still requires at least 5 months of treatment. However, the fundamental physiological mechanism inducing rudimentary embryos in *E. senticosus* species is still unclear.

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The treatment for breaking dormancy varied with plant species. Commonly, the conditionally-used aids for inducing germination are the treatments of low (15℃) and alternating temperature and chemical additives (such as GA_3) (Hudson et al., 1990). *Trollius ledebouri* (Ranunculaceae family) has rudimentary zygotic embryos in the ripen fruit. When the seeds were placed on moistened filter paper at 20℃, only 18% of germination percentage was observed after 190 days, but the testa removal or treatment with GA_{4+7} could stimulate its germination, and the germination rates were 90% and 65%, respectively after 30-day culture (Hepher and Roberts 1985 a,b). For rudimentary embryos germination of *Ilex paraguariensis*, it required a minimum of 5-9 months with appropriate environmental condition (Sansberro et al., 1998). However, when the rudimentary embryos isolated from seeds were cultured *in vitro* on LS (Linsmaier and Skoog, 1983) medium with 4% sucrose in darkness, 75% of the embryos reached maturity after 6-8 weeks of culture (Hu 1975; 1976). Sansberro et al. (1998) investigated the morphogenic response of *in vitro* cultured embryos of this species influenced by the addition or deletion of five different cytokinins, and reported that conversion of heart-shaped embryos to seedlings was achieved at about 55% after 28 days of culture, when the medium was supplemented with low concentration of ZEA ($4.5X10^{-7}$ M).

The present study was undertaken to establish the *in vitro* culture methods to understand the dormancy of zygotic embryos and to find the optimum conditions for accelerating the maturation and germination of rudimentary zygotic embryos of *E. senticosus*.

Materials and Methods

E. senticosus Maxim. seeds were collected from the Nursery, College of Forest Sciences, Kangwon National University. They were stratified in moist sand and kept at 1 5℃ for 4 weeks. These seeds were used for this experiment. To culture seeds aseptically, the dehusked seeds were sterilized in 70% alcohol for 1 minute and in 2.0% sodium hypochlorite solution for 20 minutes, and rinsed three times with sterile water.

Endosperm removal

To test the effect of endosperm removal on maturation of zygotic embryos, the endosperms at chalazal sides nourishing rudimentary embryos were removed by cross section in the scale of 1/4, 1/3 or 1/2 of seed (control is the whole seed), respectively. The excised seeds comprising zygotic embryos were placed their sides down on 1/2 MS (Murashige and Skoog 1962) medium with 1.0% sucrose on plastic Petri dishes (120×15 mm) containing 30 ml medium. The pH of medium was adjusted to 5.7, before medium was autoclaved at 121℃ for 15 min. About 20 explants were cultured on each Pertri dish and the experiment was repeated three times.

The excised seeds (1 mm-length containing zygotic embryos) with 3/4 of their endosperm removed, containing zygotic embryos, were cultured on different strengths of MS medium (1/4 X, 1/2 X, 1 X and 2 X), or on 1/2 MS medium with different sucrose concentrations (0, 1.0, 3.0 and 5.0%), or on 1/2 MS medium with different types and growth regulators (GA_3, IAA and IBA) and charcoal (0.2%), respectively. These explants were cultured on Petri dishes (150 ×20 mm) containing 30 ml medium. Twenty explants were cultured on each Petri dish and this was repeated three times. Growth of embryos was monitored after 40 days of culture.

Germination of post-mature zygotic embryos

Cotyledonary zygotic embryos that did not germinate on previous medium were transferred to 1/2 MS solid medium with 1.0% sucrose, or with 2.0 mg/l GA_3, or 0.2% charcoal for germination. After two weeks of culture, germination of embryos was monitored. Zygotic embryos were cultured on Petri dishes (150×20 mm) containing 30 ml medium. Twenty explants were cultured on each Petri dish and this was repeated three times.

Culture condition

The cultures were performed at 25±2℃ with 6/8 h (day/night) photoperiod with light intensity of about 50 μmol m^{-2} s^{-1} using white florescent tubes. All results were statistically determined, representing mean ±SE.

Results

Effect of endosperm on maturation of zygotic embryos

When *E. senticosus* seeds were stratified in moist-sand at 15℃ for one month, they slightly swelled and the zygotic embryos reached heart- shaped stage at about 0.5 mm in length. In the culture of the whole seed without testa, maturation of zygotic embryos was very slow and was similar to the growth of naturally stratified seeds, and the zygotic embryos did not show any conspicuous growth even

after 40 days of culture (Figs. 1, 2A). When the intact seeds were cultured on medium with GA_3, GA_3 alone seemed to be ineffective for growth of rudimentary embryos (data not shown).

To understand the influence of endosperm on the growth of embryos, we adopted the *in vitro* culture of seeds and analyzed the influence of endosperm on the maturation of zygotic embryos. Endosperm tissue at the chalazal side was removed carefully and the remained seeds containing zygotic embryos were cultured on 1/2 MS solid medium with 1.0% sucrose (Fig. 2). Growth and development of rudimentary embryos were significantly accelerated after the endosperm removal (Figs. 1, 2). Furthermore, as the endosperm removal of seeds increased, the growth of zygotic embryos became faster (Figs. 1, 2). It was obvious that the maximum removal of endosperm enhanced the growth and development of embryo. The average size of zygotic embryos in excised seeds with 1/2, 2/3 and 3/4 of their endosperm removed was 0.63 cm, 1.23 cm and 1.73 cm, respectively after 40 days of culture (Fig. 1). When the excised seeds with 3/4 of their endosperm removed were cultured on 1/2 MS medium with 1.0% sucrose for 40 days, almost 100% of embryos grew up to cotyledonary stage (data not shown).

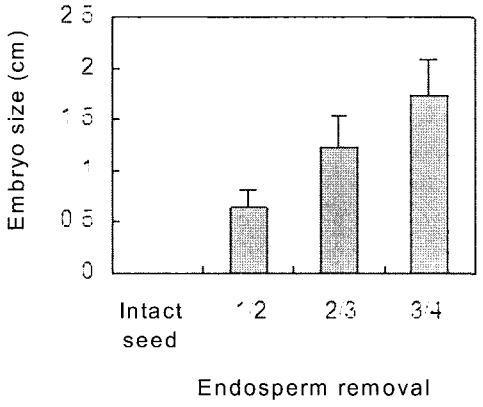

Figure 1. Growth of zygotic embryos with different size of endosperm removal on 1/2x MS medium after 40 days of culture.

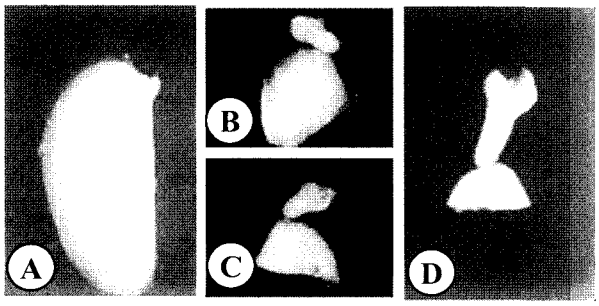

Figure 2. Effect of endosperm removal on the growth of zygotic embryos in 1/2x MS medium with 1.0% sucrose after 40 days of culture. A, B, C and D, Intact seed and seeds with 1/2, 2/3, and 3/4 of their endosperm removed, respectively.

Effect of medium, sucrose and hormone treatment

To optimize the growth of zygotic embryos by modification of culture condition, excised seeds with 3/4 of their endosperm removed were cultured on 1/4, 1/2, 1 and 2 MS medium with 1.0% sucrose, or 1/2 MS medium with 0%, 1.0%, 3.0% and 5.0% sucrose for 40 days. Growth of zygotic embryos was influenced by the strength of MS salts and concentrations of sucrose (Figs. 3, 4, 5). The growth of zygotic embryos was faster on half-strength MS medium with 1.0% sucrose than full-strength MS medium with higher sucrose concentration (Figs. 3, 4).

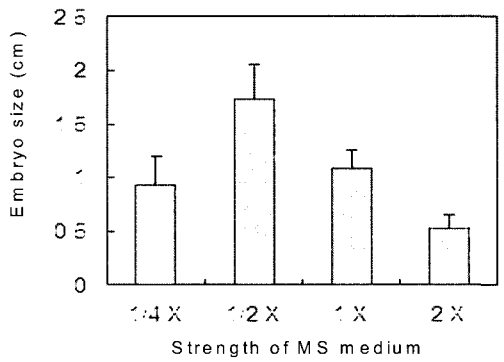

Figure 3. Growth of zygotic embryos of seeds, after 3/4 of their endosperm was removed, on different strength of MS medium after 40 days of culture.

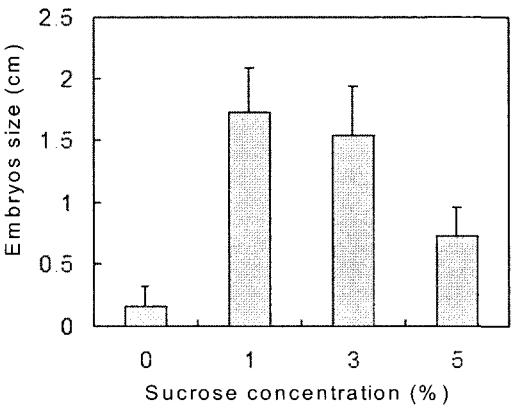

Figure 4. Growth of zygotic embryos of seeds with 3/4 of their endosperm removed on 1/2x MS medium with different sucrose concentration after 40 days of culture.

Post-mature dormancy of zygotic embryos

Zygotic embryos contained in the part of endosperm developed cotyledons. However, they did not germinate and remained in white color (Fig. 5). The post-mature dormancy of zygotic embryos was observed in all embryos regardless of the concentration of sucrose and medium (Fig. 5). Auxin treatment was less effective for the maturation of zygotic embryos than hormone-free medium (Fig. 6). The average size of zygotic embryos on medium with 2.0 mg/L IAA or 2.0 mg/L IBA were 1.06 and 0.68 cm, respectively, and the embryos were smaller than those developed on hormone-free medium (1.73 cm) (Fig. 6). On medium with GA_3 or charcoal, zygotic embryos germinated without dormancy of embryos (Fig. 7).

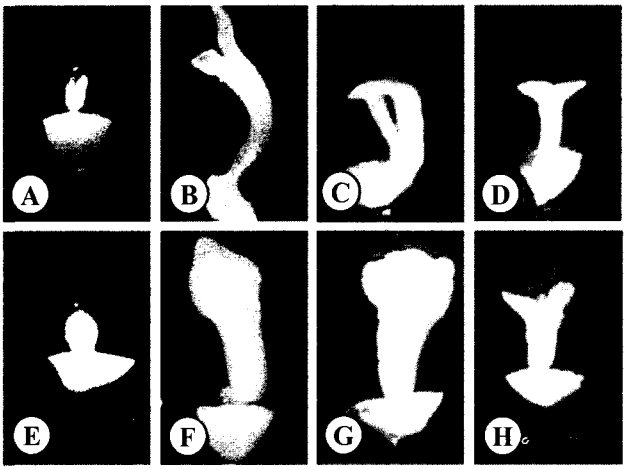

Figure 5. Growth of zygotic embryos of seeds with 3/4 of their endosperm removed after 40 days of culture. A-D, Zygotic embryo on 1/4x, 1/2x, 1x, and 2x MS medium with 1.0% sucrose, respectively; E-H, Zygotic embryo on 1/2x medium with 0, 1.0%, 3.0%, 5.0% sucrose, respectively.

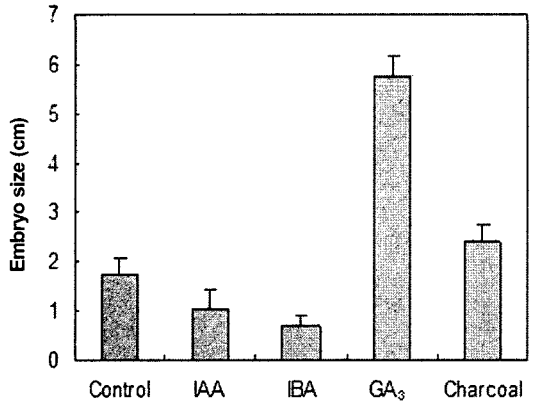

Figure 6. Growth of zygotic embryos of seeds with 3/4 of their endosperm removed on 1/2x MS medium with different growth regulators or charcoal after 40 days of culture.

Germination of zygotic embryos

The dormant cotyledonary zygotic embryos of *E. sentocosus* were separated from endosperm tissue, and were transferred onto 1/2 MS solid medium with IAA, IBA, GA_3 (2.0 mg/L) or charcoal (0.2%). The zygotic embryos detached from endosperm did not germinate on medium without hormone and with (IAA or IBA) (Figs 9A, B). The average size of zygotic embryo was the same as the original size (1.80 cm in length) of embryos after three weeks of culture (Fig. 9A-3). In contrast, zygotic embryos rapidly germinated and grew into normal seedlings on medium with GA_3 or charcoal after two weeks of culture (Fig. 9B, C), and the

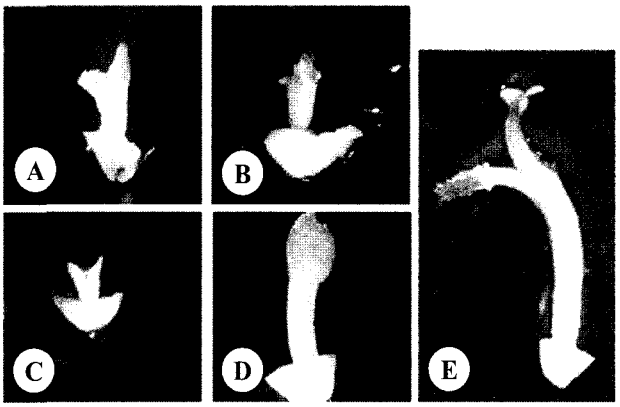

Figure 7. Growth and development of zygotic embryos of seeds with 3/4 of their endosperm removed after 40 days of culture, A, Zygotic embryo on 1/2x MS medium without any regulator; B-E, Zygotic embryos on 1/2x MS medium with IAA (2.0 mg/L), IBA (2.0 mg/L), charcoal (0.2%) and GA_3 (2.0 mg/L), respectively.

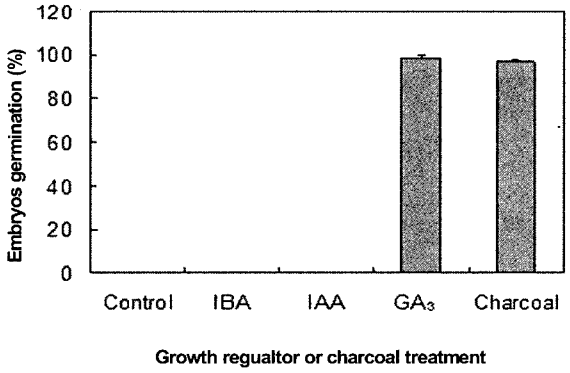

Figure 8. Germination of post-mature embryos on 1/2x MS medium with different growth regulators (2.0 mg/L each) or charcoal (0.2%) after two weeks of culture.

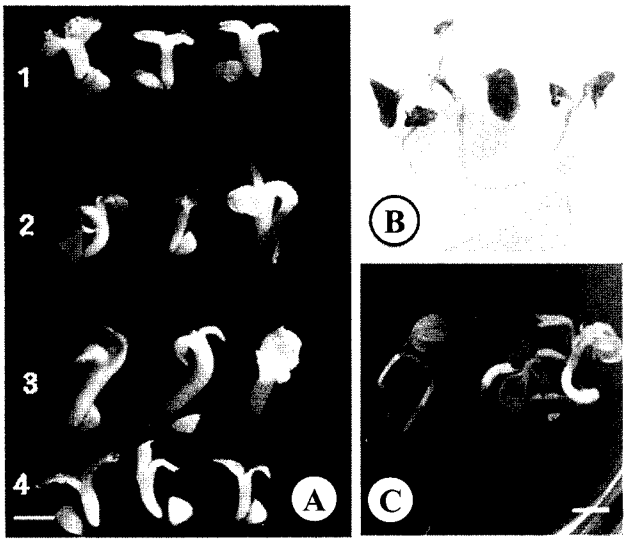

Figure 9. Growth and germination of embryos after two weeks of subculture. A: Mature embryos: A-1, 2, 3 and 4, Embryos subcultured on 2x MS, 1x MS, 1/2x MS and 1/4x MS, respectively (bar: 2mm); C-D, Rapid germination of embryos on 1/2x MS with GA_3 (2.0 mg/L) or charcoal (0.2%) (bar: 2mm).

frequency of germination was 98.4% and 96.6%, respectively (Fig. 8). These findings indicate that mature zygotic embryo possess physiological dormancy after mature to cotyledonary stage, which might be genetically programmed and not induced from environmental stress, and GA_3 is necessary to break the post-mature dormancy of embryos.

Discussion

E. senticosus seeds contain rudimentary embryos just after fruit harvest and require a long-term stratification about 18 months at low temperature and/or GA_3 treatment to break dormancy (Park *et al.* 1997). However, it is still unclear on the mechanism of their dormancy. In the culture of seeds on hormone-free medium, both testa removal and GA_3 treatment did not stimulate the growth of rudimentary embryos and no significant growth of embryos was observed. This result indicates that GA_3 is not antagonistic to the inhibitor inducing rudimentary embryos in *E. senticosus*.

We tested the influence of endosperm on the maturation of zygotic embryos by *in vitro* culture of zygotic seeds after endosperm removal. Using *in vitro* culture of seeds, endosperm removal markedly accelerated the growth of rudimentary embryos, and the immature heart-shaped embryos were matured to cotyledonary stage embryos within one month of culture. Growth of zygotic embryos was more rapid as the endosperm tissue surrounding zygotic embryos was smaller. In natural stratification condition, several months are required for the growth of rudimentary zygotic embryos into cotyledonary stage (Zhu and Wang 1992). The rapid maturation of zygotic embryos by endosperm removal can be explained by following two factors. One is that endosperm removal facilitated the absorption of nutrient into rudimentary embryo, and embryo could more rapidly grow within the seed after endosperm removal. Another possible reason is that there are some inhibitors in endosperm, and the endosperm removal weakens the action of endosperm that suppresses embryo maturation, which results in the rapid growth of embryos. We observed that treatment of charcoal stimulated the growth of embryos. The maturation promoting effect by charcoal treatment indicates that the charcoal might absorb inhibitors in the endosperm. It was reported that charcoal could absorb aromatic molecules preferentially over straight chain ones, and the addition to medium is to remove aromatic waste products excreted by cultured tissues (Fridborg et al. 1978; Wang and Huang 1976). Based on the above points, we agree to this second suggestion that the primary factors inducing rudimentary embryo was the inhibition of endosperm tissue surrounding zygotic embryos. The rapid maturation of rudimentary zygotic embryos by endosperm removal will be applied to the other species to stimulate the maturation of zygotic embryos.

The putative inhibitor inducing rudimentary embryos was not chemically identified in all plant species. In rudimentary zygotic embryos of *Trollius ledebouri* (Ranunculaceae family), live washing (presoaking) treatments could promote the seed germination (Hepher and Roberts 1985a, b). They presumed that seed coat removal and presoaking in water directly decreased some inhibitor in endosperm, and thus lead to maturation of zygotic embryos. GA_3 can be antagonistic to putative inhibitor in seeds with rudimentary embryos. In the present investigation, we report that GA_3 has no effect in breaking dormancy and growth of rudimentary embryos and there may be some other inhibitors that may bring about dormancy, which is not known. Similar result was shown in *Panax quinquefolium*, a relative genus to *E. sentocosus*, and Zhao et al. (2000) considered that exogenous GA_3 could not be used to accelerate the growth of immature embryo, but might help in relieving seed dormancy during physiological after-ripening period.

In the culture of excised seeds after endosperm removal, almost 100% embryos matured rapidly to cotyledonary stage, but further growth for the germination did not occur. The germination of embryo was not overcome by MS medium concentration and different sucrose concentrations. However, germination of zygotic embryos occurred rapidly within two weeks of culture when excised seeds were cultured on medium with GA_3 or charcoal. Choi et al. (1999)

reported that somatic embryos developed from the embryogenic callus of *E. senticosus* have similar dormancy although they grow on *in vitro* culture condition, GA_3 was necessary to stimulate the germination of somatic embryos similar to naturally grown zygotic seeds, and the dormant embryos contain higher concentration of endogenous ABA. The effect of GA_3 on dormancy breaking has been extensively reported in lots of plants (having physiology dormancy). The action of the dormancy breaking is generally related to the levels of endogenous GA_3 and ABA in seed (Bewley 1997). These results indicate that zygotic embryos might be in physiologically dormant after mature to cotyledonary stage even though they were free from endosperm tissue and cultured in *in vitro* culture condition. This indicates another dormancy of post-mature zygotic embryos.

In conclusion, through the method of *in vitro* culture of excised *E. senticosus* seeds after endosperm removal, we found that zygotic embryos of *E. senticosus* not only have morphological dormancy (rudimentary dormancy) influenced by endosperm tissue, but also have physiological dormancy after post-mature embryos. *In vitro* culture of excised seeds with their endosperm removal accelerated the growth and maturation of embryos. GA_3 (2.0 mg/L) or charcoal (0.2%) effectively breaks the physiological dormancy of post-mature embryo, leading to the significant transition of embryo to seedling by GA_3 treatment. The combined activity of endosperm removal and GA_3 treatment is effective and simple for the rapid germination of zygotic embryos.

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