Plant Cell Rep (2002) 20:1112–1116
DOI 10.1007/s00299-002-0455-y

CELL BIOLOGY AND MORPHOGENESIS

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Dormancy induction of somatic embryos of Siberian ginseng by high sucrose concentrations enhances the conservation of hydrated artificial seeds and dehydration resistance

Received: 10 November 2001 / Revised: 28 January 2002 / Accepted: 29 January 2002 / Published online: 4 April 2002

Abstracts In most plants, somatic embryos tend to germinate prematurely, a process that is detrimental to controlled plant production and the conservation of artificial seeds. We investigated the dormancy characteristics of Siberian ginseng somatic embryos induced simply by a high sucrose treatment, a treatment that enables the long-term conservation of artificial seeds following encapsulation and provides embryos with an enhanced resistance to dehydration. Early-cotyledonary stage somatic embryos were mass-produced by means of bioreactor culture. These embryos were then plated on medium supplemented with various levels of sucrose (1%, 3%, 6% or 9%) and allowed to mature. Subsequent germination of these embryos following the maturation period depended significantly on the sucrose level. At concentrations of 9% sucrose, none of the somatic embryos germinated after maturation, and none were recovered after being transferred to half-strength MS medium containing 2% sucrose. Gibberellic acid treatment was necessary to induce germination; other growth regulators such as auxins and cytokinins did not induce a response. Endogenous abscisic acid content in somatic embryos matured at 9% sucrose (487.8 ng/g FW) was approximately double that found in those matured at 3% sucrose (258.4 ng/g FW). This indicates induced dormancy in embryos under high osmotic stress. Alginate encapsulation of embryos facilitated the artificial induction of dormancy to extend the conservation period without germination. The induction of dormancy strengthened resistance to dehydration after the embryos were desiccated to 15% of their normal water content. Reduced chances of embryo survival during long-term desiccation were distinctly delayed in dormant embryos. These results indicate that the induction of dormancy in embryos is a promising application for synthetic seed production.

Communicated by F. Sato

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Keywords Large-scale culture · Artificial seeds · Encapsulated embryos

Abbreviations *ABA*: Abscisic acid · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · GA_3: Gibberellic acid

Introduction

Seed dehydration accompanied by the maturation of zygotic embryos results in the dormancy of zygotic embryos as the flower organ ripens. Dormancy of zygotic embryos is useful in propagating plants, particularly for synchronous germination and long-term storage. In contrast, premature germination is the major problem in somatic embryogenesis, with the result that the conservation and germination of somatic embryos are barely controllable. In hydrated alginate capsules containing somatic embryos, some of the problems connected to the long-term conservation of beads occur because of unnecessary germination during conservation (Gray et al. 1987). These encapsulated somatic embryos could be stored at low temperatures for only a few weeks (Fuji et al. 1992). The desiccation of somatic embryos is an ideal step in the long-term conservation of somatic embryos only if the dehydration of somatic embryos is not detrimental to survival, as was tested by Gray et al. (1987). While various stress treatments have been shown to increase the survival of desiccated somatic embryos (Kim and Jonick 1989; Mckersie et al. 1989), the desiccation tolerance of somatic embryos is not yet at the level tolerated by zygotic embryos.

Siberian ginseng (*Eleutherococcus senticosus*) is an endangered medicinal woody plant species. Conventional propagation is very difficult because of the long-term stratification required to induce both maturation and germination of the zygotic embryos (Isoda and Shoji 1994). The low rooting frequency of cuttings likewise hinders propagation. Plant regeneration of Siberian ginseng through direct somatic embryogenesis (Choi et

al. 1999c; Gui et al. 1991) and indirect embryogenic callus and cell suspension culture (Choi et al. 1999b) has been reported.

In this paper, we report that the artificial induction of dormancy in somatic embryos by a high sucrose treatment increases desiccation tolerance and long-term conservation of alginated capsules without germination.

Materials and methods

Somatic embryo induction

Embryogenic cells of Siberian ginseng (*Eleutherococcus senticosus* Harms) were induced from excised zygotic embryos using the protocol of Choi et al. (1999b). Embryogenic cells were maintained in MS (Murashige and Skoog 1962) liquid medium containing 3% sucrose and supplemented with 1.0 mg/l 2,4-D in 250-ml Erlenmeyer flasks with subculture at 2-week intervals. The mass production of somatic embryos was achieved by transferring 2 g of embryogenic cells to a 3-l air-bubble glass bioreactor containing 2 l MS liquid medium with 3% sucrose but without 2,4-D. The embryos formed in each bioreactor were separated into three bioreactors at 2-week subculture intervals to support the development of the somatic embryos. The bubble generating system at the bottom of the tank was equipped with a sintered metal. Air pressure was adjusted to a flow rate of 0.05–0.1 vvm [volume (aeration) per volume (medium) per minute]. The media were sterilized in 121°C for 15 min after the pH had been adjusted to 5.8. The culture room was maintained at 22°±2°C and a 16/8-h (day/night) photoperiod with light supplied by white fluorescent tubes at an intensity of 24 µmol m^{-2} s^{-1}.

Somatic embryos at the early-cotyledonary stage were plated on the surface of MS solid medium (0.25% Gelrite) containing various levels of sucrose (1%, 3%, 6% or 9%) in 10×2-cm petri dishes. Each dish contained about 100 embryos. Five replicates were used per treatment, and the experiment was repeated three times. After 1 month, the germination frequency of the embryos was investigated. The culture room was maintained as for the bioreactor culture.

Cotyledonary-stage somatic embryos that failed to germinate due to high sucrose concentrations (6% and 9%) were transferred to half-strength MS solid medium containing 1% sucrose with or without 2 mg/l GA_3 to induce germination. After 3 weeks, the germination frequency of the embryos was observed.

ABA analysis

ABA was extracted from somatic embryos cultured in media with 3% and 9% sucrose as described by Mertens et al. (1983). Competitive ELISA analysis was performed to quantify endogenous ABA, and the procedures of Phytodetek ABA kit (Agdia, Ind.) for the ABA ELISA were followed. Standard curves were generated using a racemic mixture of (±) *cis/trans* ABA (Sigma, St. Louis, Mo.).

Encapsulation of somatic embryos

Cotyledonary embryos that matured at different sucrose levels (3% and 9%) were immersed in a 3% sodium alginate solution. Each embryo was then sucked up into a glass pipette with a 0.5-cm-diameter tip and transferred into a 100 m*M* $CaCl_2 \cdot 2H_2O$ solution drop by drop. After 10 min, the alginated capsules were washed using fresh, hormone-free MS liquid medium. About 20 capsules were transferred to half-strength MS solid medium containing 2% sucrose with or without 2 mg/l GA_3 in a 10×2-cm plastic petri dish. The germination of encapsulated somatic embryos was investigated after 2 weeks and 10 weeks of culture.

Desiccation of somatic embryos

Somatic embryos that matured in media containing 1%, 3%, 6% or 9% sucrose with or without 1.0 mg/l ABA were placed on the surface of sterilized filter paper in plastic petri dishes and kept unsealed. The petri dishes were kept in the dark at 70% humidity in the growth chamber for about 2 days until the water content of the embryos reached only 15%. Thereafter, the petri dishes were sealed with Parafilm to prevent any further dehydration of the embryos. The water content of the embryos was measured by weight lost. These desiccated embryos were kept for 1–9 weeks in the dark at 22°C and then transferred to half-strength MS solid medium containing 2% sucrose and 2 mg/l GA_3. The survival and conversion of the somatic embryos into plants were determined after 1 month.

Results and discussion

Large-scale production of somatic embryos

Two grams of embryogenic cells maintained in a 250-ml Erlenmeyer flask was transferred to 3-l air-bubble bioreactors containing 2 l of MS medium with 3% sucrose and lacking 2,4-D (Fig. 1A). Torpedo-shaped embryos were formed after 1 month of culture (Fig. 1B) and early-cotyledonary stage embryos formed after an additional 4 weeks of culture (Fig. 1C). When these cotyledonary embryos were plated on half-strength MS agar medium containing 1% sucrose, germination occurred after 1 month (Fig. 1D). About 12,000 embryos were produced in a bioreactor. All of the embryos developed independently and in a synchronized state without any special sieve and synchronization treatment of the embryogenic cells. No conspicuously elongated non-embryogenic cells were detected among the suspension. This result indicates that the embryogenic competency of Siberian ginseng cells is very high and that they can be used as a model system in somatic embryogenesis research.

Induction of dormancy in somatic embryos by high sucrose levels

Early-cotyledonary stage embryos were harvested from the bioreactor and plated on MS solid medium containing 1%, 3%, 6% or 9% sucrose for 1 month. The germination process was highly dependent on the level of sucrose in the medium. At 1% sucrose, 97% of the somatic embryos matured to green cotyledonary embryos and germinated without any resting event (Figs. 2A, B; 3). At 9% sucrose, embryos were opaque-white in color and did not germinate following maturation (Figs. 2C, D; 3). The resting state of embryos continued even after being transferred to half-strength MS medium with a low sucrose level (1%). However, the resting embryos germinated rapidly and converted into plantlets when they were transferred to a medium supplemented with 2 mg/l GA_3. Other types of growth regulators such as auxins (indole-3-acetic acid or indole-3-butyric acid) or cytokinins (kinetin or 6-benzylaminopurine) were not responsive (data not shown). The GA_3 requirement of the dormant somatic embryos closely

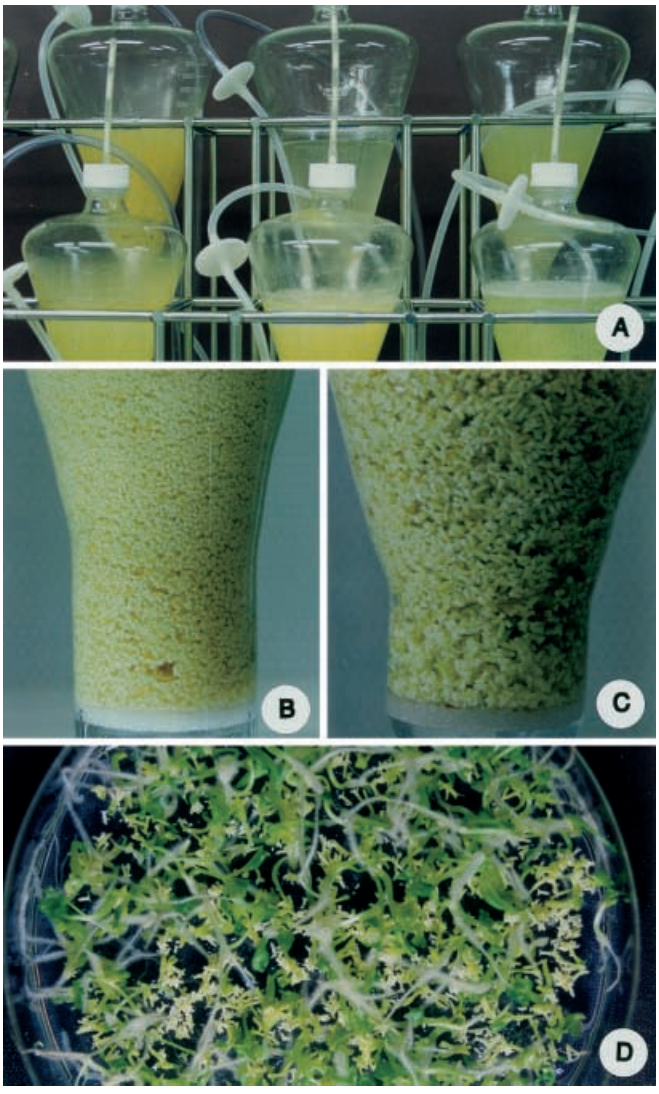

Fig. 1A–D Mass production of somatic embryos of Siberian ginseng through bioreactor culture. **A** Production of somatic embryos by means of 3-l bioreactor culture. **B** Heart-shaped somatic embryos 3 weeks after the transfer of embryogenic cells to MS medium supplemented with 3% sucrose. **C** Torpedo embryos after 1 month of culture. **D** Germination of somatic embryos following plating on the surface of half-strength MS agar medium supplemented with 1% sucrose

resembles the dormancy-breaking treatment of zygotic seeds, indicating that Siberian ginseng somatic embryos manifest the dormancy phenomenon under high osmotic stress. In zygotic seeds of Siberian ginseng, a stratification treatment of about 1.5 years was required to induce germination (Isoda and Shoji 1994).

Since the induction of dormancy in zygotic embryos has been found to be related to the accumulation of endogenous ABA (Bewley 1997), we analyzed endogenous ABA content in somatic embryos of *E. senticosus* treated with 3% and 9% sucrose. The level of endogenous ABA (487.8 ng/g FW) in the somatic embryos treated with 9% sucrose was approximately double that of those treated with 3% sucrose (258.4 ng/g FW).

These results indicate that a high osmotic treatment might stimulate somatic embryos of *E. senticosus* into dormancy. Choi et al. (1999a) observed a similar dormant phenomenon after applying a high sucrose treatment to somatic embryos of *Panax ginseng*. However, in most plants, premature germination is the main problem, while ABA and high sucrose treatments are used only to temporarily suppress embryo germination (Ammirato 1977).

Gray (1987) suggests that the term “quiescence” of embryos designates a resting state reversed solely by the addition of water, whereas “dormancy” refers to a resting state where the resumption of growth is dependent upon a specific treatment or condition in addition to the presence of water. In *E. senticosus*, special dormancy-breaking treatments, such as the presence of GA_3, are required to induce the germination of resting embryos, thus indicating that the embryos of *E. senticosus* following high sucrose treatment are in the dormant state.

Encapsulation of somatic embryos

In general, artificial seeds cannot be conserved for a long period because of unnecessary germination even at low temperatures. In alfalfa, hydrated encapsulated embryos could be stored at low temperatures for only a few weeks (Fujii et al. 1992; Redenbaugh et al. 1986). When somatic embryos of Siberian ginseng encapsulated with calcium alginate were cultured on half-strength MS medium containing 2% sucrose, 97% of those matured at 3% sucrose turned green and germinated within 3 weeks. Of the encapsulated embryos matured at 9% sucrose, 100% did not germinate and their white color was retained up to 2 months of culture (Fig. 2E). The alginated capsules germinated readily after transfer to a medium supplemented with 2 mg/l GA_3 (Fig. 2F). This means that inducing dormancy in somatic embryos is highly advantageous for long term conservation and controlled germination of artificial seeds.

Desiccation of somatic embryos

The resistance of somatic embryos to dehydration may be promising for dry artificial seed production, as suggested by Gray et al. (1987). Thus, the survival of somatic embryos of Siberian ginseng following a desiccation treatment was investigated. Somatic embryos that matured at 3% and 9% sucrose and/or 1 mg/l ABA were dehydrated on sterilized filter paper until the water content fell below 15%. In dehydrated, non-dormant embryos that matured at 1% sucrose, none of the embryos survived after dehydration (Fig. 4). In dehydrated embryos that matured at 3% sucrose, only 17% embryos were converted into plantlets after 1 week of dehydration (Fig. 2G). Radicles of embryos was more sensitive to dehydration stress than the shoot apical meristem part – some somatic embryos that matured at 3% sucrose

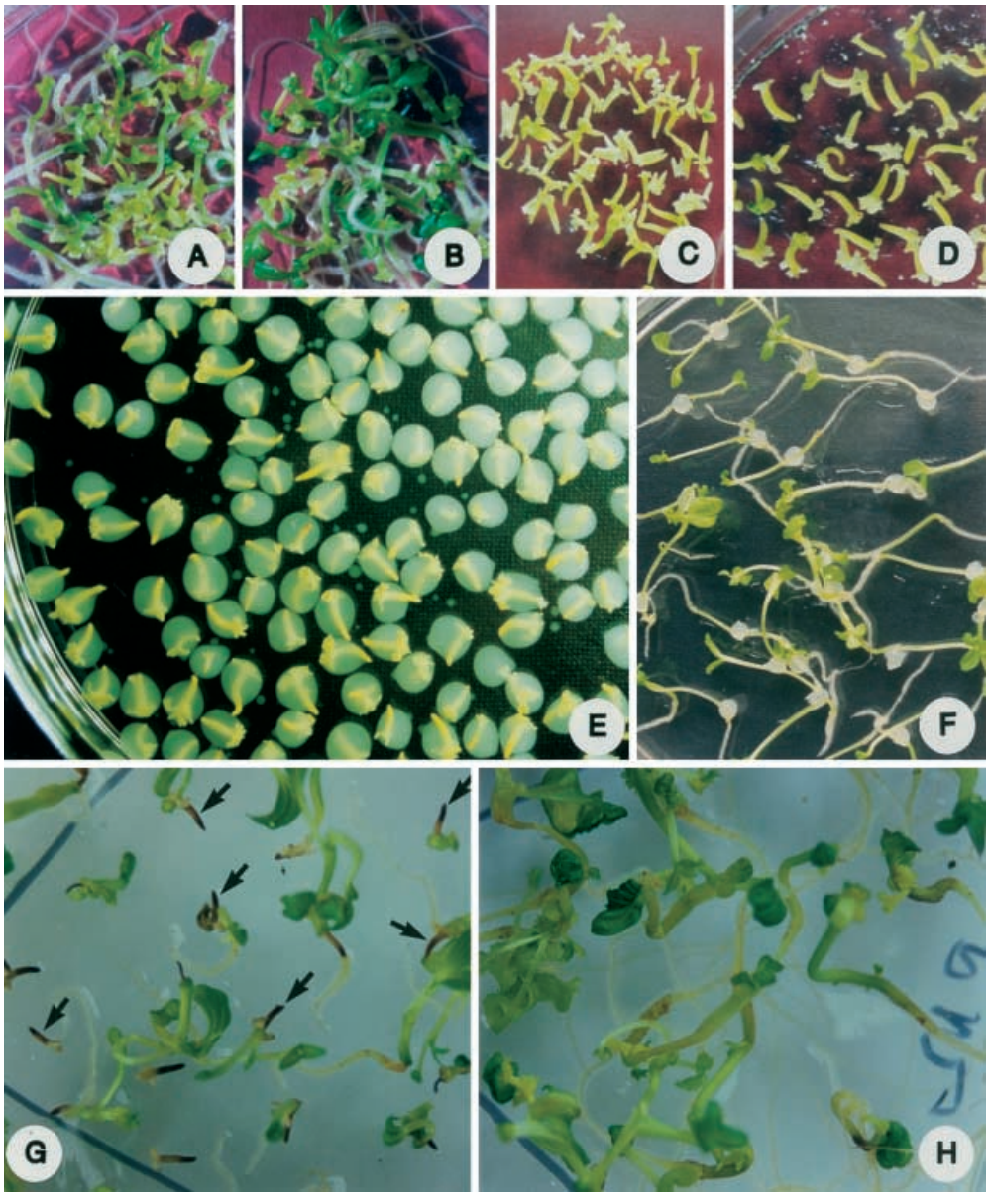

Fig. 2A–H Germination of encapsulated artificial seeds and dehydrated somatic embryos of Siberian ginseng that were matured at different sucrose levels. **A**, **B** Germination of somatic embryos matured at 3% sucrose after 3 weeks (**A**) and 1 month (**B**) of culture. **C**, **D** Somatic embryos without germination that were matured at 9% sucrose after 3 weeks (**C**) and 1 month (**D**) of culture. **E** Encapsulated artificial seeds containing somatic embryos matured at 9% sucrose after 1 month of encapsulation. **F** Germination of encapsulated artificial seeds containing embryos that had matured at 9% sucrose, following their transfer to MS medium supplemented with 2 mg/l GA_3. **G** Low survival of dehydrated somatic embryos matured at 3% sucrose. *Arrows* indicate browned hypocotyls and radicles damaged by dehydration. **H** High survival of dehydrated somatic embryos matured at 9% sucrose

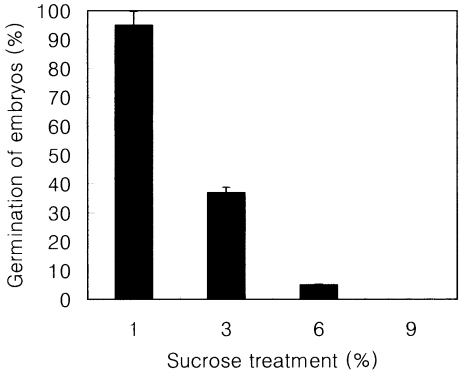

Fig. 3 Germination of somatic embryos on MS medium supplemented with different concentrations of sucrose after 1 month of culture. The culture room was maintained at 22°±2°C and a 16/8-h (day/night) photoperiod with light supplied by white fluorescent tubes at an intensity of 24 µmol m^{-2} s^{-1}

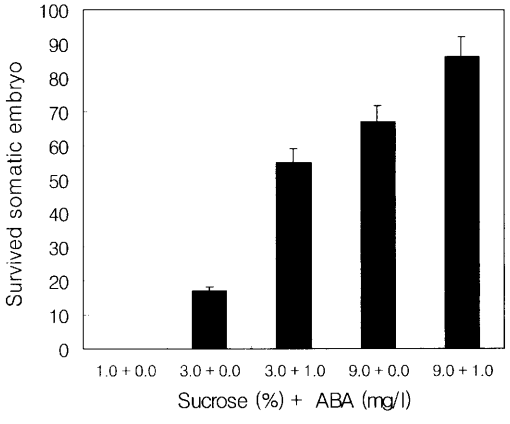

Fig. 4 Effect of the combined sucrose and ABA pretreatment on the survival of somatic embryos after 1 week of dehydration

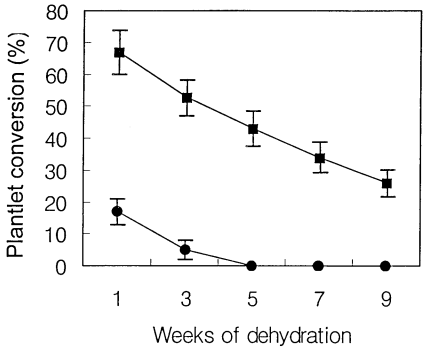

Fig. 5 Decline of survival of somatic embryos pretreated with 3% (*black circle*) and 9% sucrose (*black box*) during the 9 weeks of dehydration

produced shoots despite the browning of the radicle part (Fig. 2G, arrows). In contrast, root emergence without plumule emergence is common in alfalfa (Lai et al. 1995). High sucrose (9%) treatment strongly stimulated the resistance of embryos to dehydration: 67% of the dehydrated embryos that had matured under conditions of 9% sucrose germinated and were converted into plantlets (Figs. 2H, 4). The 9% sucrose treatment combined with 1 mg/l ABA treatment was even more effective in increasing resistance to dehydration (Fig. 4): 86% of the dehydrated embryos were recovered after rehydration in embryos treated with both 9% sucrose and ABA (Fig. 4). When somatic embryos were treated with 1.0 mg/l ABA alone, the result resembled that observed when the high sucrose treatment was applied. However, ABA treatment stimulated callus production from root tips and/or secondary somatic embryogenesis on the surface of the embryo axis (data not presented). Therefore, plant conversion from ABA-treated embryos was somewhat abnormal with secondary embryos on the plantlet bodies. In wheat somatic embryos, ABA treatment promoted embryogenic callus induction (Javed et al. 1989).

The decline in survival of dehydrated embryos pretreated with 3% and 9% sucrose as time elapsed was investigated (Fig. 5). In dehydrated embryos that matured at 3% sucrose, only 17% of the embryos were converted into plantlets 1 week after dehydration, and all of the embryos died by 5 weeks of dehydration. Of the embryos that matured at 9% sucrose, 67% survived 1 week after dehydration, and survival declined to 26% after 9 weeks (Fig. 5).

These results indicate that the artificial induction of dormancy by a high sucrose treatment strongly enhanced the conservation of encapsulated artificial seeds and enhanced the resistance of somatic embryos to dehydration. If some suitable coating for dried somatic embryos and an artificial endosperm capsule were to be developed, somatic embryos might be able to be conserved for a long period and used as dried-type artificial seeds.

Acknowledgements This work was supported by a research grant from the Korean Ministry of Science and Technology (01-J-BP-01-B-57).

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