

# Inhibitory Factor(s) of Somatic Embryogenesis Regulated Suspensor Differentiation in Suspension Culture of Japanese Larch (*Larix leptolepis* GORDON)

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Received 20 January 2003; Accepted 18 August 2003

## Abstract

Using somatic embryogenesis system of Japanese larch that develops embryos composed of embryo-proper and suspensor, the effects of high-cell-density culture and conditioned medium on the somatic embryogenesis were investigated. High-cell-density culture strongly inhibited the somatic embryogenesis. Furthermore, the conditioned medium derived from high-cell-density culture also strongly inhibited the somatic embryogenesis, especially differentiation of the suspensor. The inhibitory effect of the conditioned medium was not attributable to the depletion of nutrients, but to the accumulation of inhibitory factor(s) in the medium. The addition of activated charcoal to high-cell-density culture resulted in the formation of numerous somatic embryos with longer suspensors than in the untreated one. This treatment also resulted in the formation of numerous vacuolated cells-like suspensor on the surface surrounding the embryo-proper. These results indicate that some inhibitory factor(s) that regulates suspensor differentiation are released into the medium of high cell density.

**Key words:** embryo-proper, inhibitory factor, Japanese larch, somatic embryogenesis, suspensor.

## Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; 4HBA, 4-hydroxybenzyl alcohol; ABA, abscisic acid; BAP, benzyl amino purine; EC, embryogenic cells; HCM, conditioned medium in which EC has been cultured for three weeks at high cell density (5.0 ml PCV l<sup>-1</sup>); mCD medium, modified Campbell and Durzan's medium; PCV, packed cell volume.

## Introduction

Various environmental and chemical factors influence cell proliferation and morphogenesis in plant tissue culture. Initial cell density is one of the important factors in liquid suspension culture. In general, suspension cultured cells proliferate actively at high cell density, and dilution of the cell culture markedly decreases mitogenic activity of the cells. Addition of culture filtrate (conditioned medium), in which cells have proliferated actively,

increases mitogenic activity of the cells. Cells can proliferate rapidly even in low-cell-density culture by the addition of such conditioned medium (conditioning effect) (Bellincampi and Morpurgo, 1987, 1989; Birnberg *et al.*, 1988; Schroder *et al.*, 1989; Hagimori and Nagaoka, 1992; Jorgensen *et al.*, 1992). Cell density and conditioning are also important for the induction of somatic embryogenesis. It was previously reported that secreted phyto-sulfokine and arabinogalactan proteins into culture medium stimulated its somatic embryogenesis (Kobayashi *et al.*, 1999b; Hanai *et al.*, 2000; van Hengel *et al.*, 2001). On the other hand, high-cell-density culture strongly inhibited somatic embryogenesis (Fridborg and Eriksson, 1978; Sung and Okimoto, 1981; Osuga *et al.*, 1993). This inhibition is attributable to chemical substances released from cultured cells into the medium but not to nutrient depletion or physical damage (Higashi *et al.*, 1998). The inhibitory conditioning factor(s) specifically inhibits rapid cell division that is characteristic of

the early stage of somatic embryogenesis (Kobayashi *et al.*, 1999a). Recently, one of the inhibitory factors was purified and identified as 4-hydroxybenzyl alcohol (4HBA) (Kobayashi *et al.*, 2000a, b).

In *Arabidopsis*, many zygotic embryogenesis mutants have been identified, including an abnormal suspensor mutant, raspberry mutant, and twin mutant, which give rise to extra embryos from the suspensor (Schwartz *et al.*, 1994; Vernon and Meinke, 1994; Yadegari *et al.*, 1994). These suspensor mutants are recessive and the development of the embryo-proper is abnormal in all identified mutants. These mutants imply that an inhibitory factor(s), which suppresses the embryogenic potential of suspensor cells and normal development of the suspensor, is produced by the embryo-proper (Marsden and Meinke, 1985; Yeung and Meinke, 1993; Schwartz *et al.*, 1994). To verify this hypothesis, it is important to study the factor(s) that inhibit embryogenesis in the suspensor. However, it is difficult to clarify the effect of the inhibitory factor(s) on the differentiation of suspensor, because there is no way to isolate and culture immature zygotic embryos with the suspensor. Moreover, somatic embryos of angiosperms, such as carrot and *Arabidopsis*, lack morphological and functional suspensors.

In angiosperm, the first zygotic division produces a basal cell, which forms the suspensor, and a terminal cell, which forms the embryo-proper. Gymnosperm, however, undergo a free-nuclear phase where several nuclear divisions occur before cell plate formation. Another round of division produces four-tiered, 16-celled pro-embryos. In the micropylar end of the seed, the four cells in the distal tier form the embryo proper, and the next tier form the suspensor (Ciavatta *et al.*, 2001). Although the first process to form embryo-proper and suspensor is different, it is considered that the role of suspensor in both angiosperm and gymnosperm is to support the growth of the embryo.

It is proposed that culture systems of some conifers are suited to study suspensor biology, because somatic embryos in their culture system form well-developed suspensors (Ciavatta *et al.*, 2001). Therefore, we focused on somatic embryogenesis in Japanese larch (*Larix leptolepis* GORDON). In Japanese larch, a tissue culture system for inducing somatic embryogenesis from immature zygotic embryos has been established (Ogita *et al.*, 1997). Plantlets are developed from somatic embryos upon treatment with abscisic acid (Ogita *et al.*, 1999). Recently, the effect of initial cell density on somatic embryogenesis has been investigated in the Japanese conifers *Larix leptolepis* (Japanese larch),

*Picea jezoensis*, and *Cryptomeria japonica* (Ogita *et al.*, 2000), and pro-embryos were not formed at high cell density. However, optimum cell density and conditioning effects in somatic embryogenesis are not investigated yet.

In this study, we investigated the effect of possible factor(s) released into conditioned medium derived from a high-cell-density culture on somatic embryogenesis of the Japanese larch.

## Materials and Methods

### *Plant materials and induction of pro-embryos*

Embryogenic cells (EC) of Japanese larch used in this study were prepared as described previously (Ogita *et al.*, 1997). EC were maintained by the three-week subculture in a modified Campbell and Durzan's basal medium (mCD medium) (Campbell and Durzan, 1975) supplemented with 7  $\mu\text{M}$  2,4-D, 3  $\mu\text{M}$  BAP, and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.6 before autoclaving at 121°C for 15 min. To induce the development of somatic embryos, EC (100–250  $\mu\text{m}$  in diameter) were collected by passing the culture medium through stainless-steel sieves (pore size 250 and 100  $\mu\text{m}$ ). The EC were rinsed five times with phytohormone-free mCD medium, collected by centrifugation at 100g, and suspended in 10 ml of phytohormone-free mCD medium in 100-ml flasks at cell densities of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ml packed cell volume (PCV) per litre of the medium. The cells were cultured on a gyratory shaker (100 rpm) at 25°C for 16-h photoperiod provided by fluorescent lamps (white light at 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). To compare the number of somatic embryos that formed at different initial cell densities, the number was normalised using 1 ml PCV of the initial EC.

### *Maturation of pro-embryos and regeneration of plantlets*

The EC cultures were transferred onto a solidified mCD medium containing 3  $\text{g l}^{-1}$  Gellan gum, 50  $\mu\text{M}$  ABA and 3% (w/v) sucrose. They were subcultured every three weeks, and the subculture was repeated two times to allow maturation and development of pro-embryos. The cotyledonary embryos obtained were put on a fresh phytohormone-free solidified mCD medium containing 2  $\text{g l}^{-1}$  Gellan gum and 3% (w/v) sucrose, and cultured as described above to induce plantlet formation.

### *Observation and count of somatic embryos*

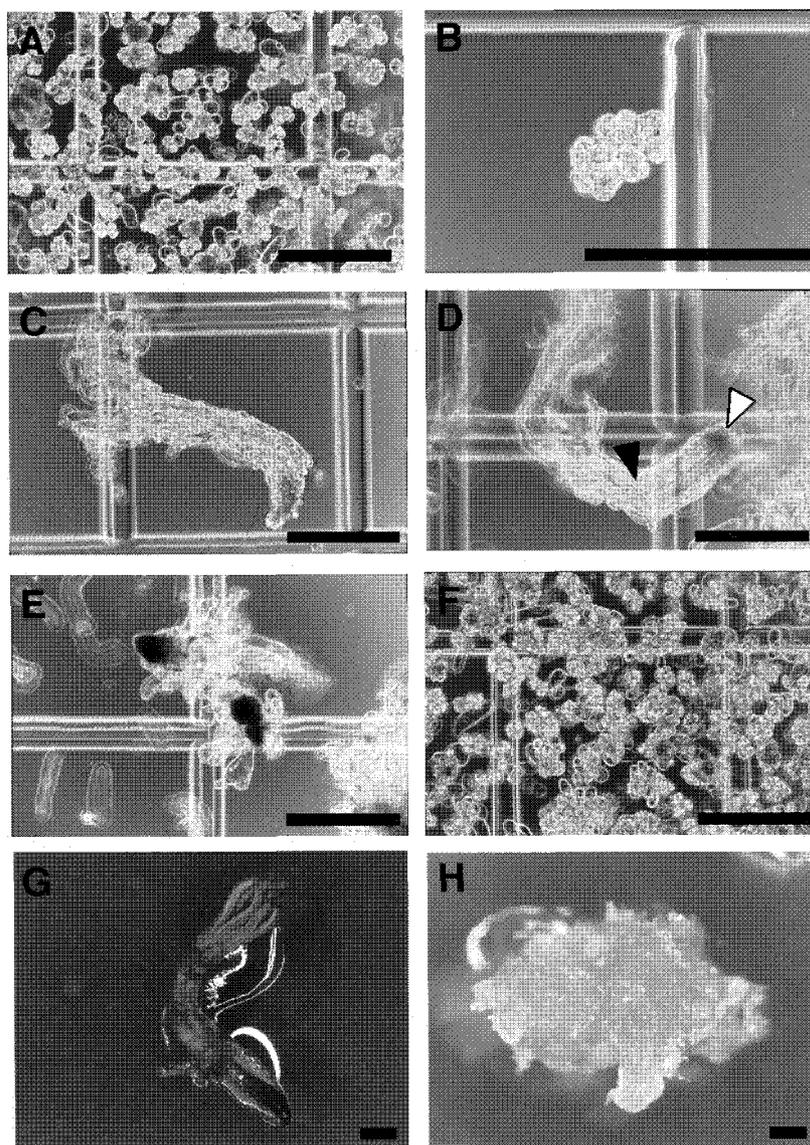
The development of suspension cells in liquid mCD medium was investigated under a phase-

contrast microscope (OLYMPUS, BX51). The number of somatic embryos formed was counted after three weeks of culture in phytohormone-free mCD medium. The experiments were repeated at least twice with five replications.

#### *Preparation and assay of conditioned medium*

Conditioned medium in which EC had been cultured for three weeks at high cell density (5.0 ml PCV l<sup>-1</sup>) (HCM) was collected by filtration through Whatman glass microfibre filters (GF/A). The filtrate was adjusted to pH 5.6 and sterilised by filtration using Whatman syringe filters (GD/X).

Zero, 2.5, 5.0, 7.5, and 10 ml of HCM was mixed with 10, 7.5, 5.0, 2.5, and 0 ml, respectively, of fresh phytohormone-free mCD medium to make a final volume of 10 ml. Note that the concentrations of the mCD medium nutrients were equal to or less than those of the mCD medium. EC were suspended in each of these test media at a cell density of 0.5 ml PCV l<sup>-1</sup>. Three weeks after the culture, the number of somatic embryos was counted. Alternatively, another type of HCM-containing test media was prepared as follows. Zero, 2.5, 5.0, 7.5, and 10 ml of HCM was first mixed with 10, 7.5, 5.0, 2.5, and 0 ml, respectively, of distilled water to make a final



**Fig. 1** Morphological changes in Japanese larch cells cultured at various initial cell densities. EC at various initial cell densities were cultured for three weeks in phytohormone-free mCD medium. (A) EC at the start of culture. (B-F) Cells cultured at 0.05, 0.1, 0.5, 1.0, and 5.0 ml PCV l<sup>-1</sup>, respectively. (G) Mature plantlet resulting from ABA (50 μM) treatment of somatic embryos obtained at 0.5 ml PCV l<sup>-1</sup>. (H) Large cell clusters formed after treatment of cells obtained at 5.0 ml PCV l<sup>-1</sup> with ABA (50 μM). No somatic embryos formed on the cell clusters. White arrowhead shows embryo-proper. Black arrowhead shows suspensor. The bar indicates 500 μm.

volume of 10 ml. To 5 ml of each, 5 ml of 2x mCD medium in which all of the mCD medium nutrients were two-fold strength, was added to make a final volume of 10 ml. Note that concentrations of the mCD medium nutrients were equal or greater than those of the mCD medium. EC were suspended at a cell density of  $0.5 \text{ ml PCV l}^{-1}$ , and three weeks after the culture, the number of somatic embryos was counted. The experiments were repeated at least twice with five replications.

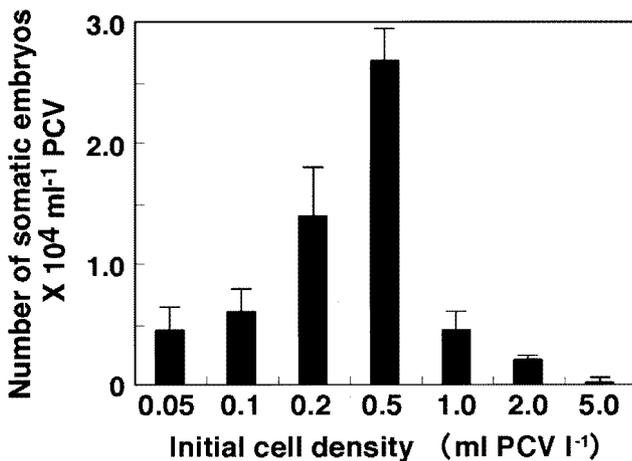
#### Addition of activated charcoal

Activated charcoal (WAKO, granular type) was added at concentrations of 0.5% and 1% (w/v) to EC liquid cultures of various cell densities ( $0.5, 1.0, 1.5, 2.0, 3.0, 4.0,$  and  $5.0 \text{ ml PCV l}^{-1}$ ) in phytohormone-free mCD medium supplemented with 3% (w/v) sucrose. The number of somatic embryos was counted after three weeks of the culture, as described above. The lengths of the embryo-proper and suspensor were measured separately using a digital HD microscope (KEYENCE, VH-7000). The experiments were repeated at least twice with five replications.

## Results

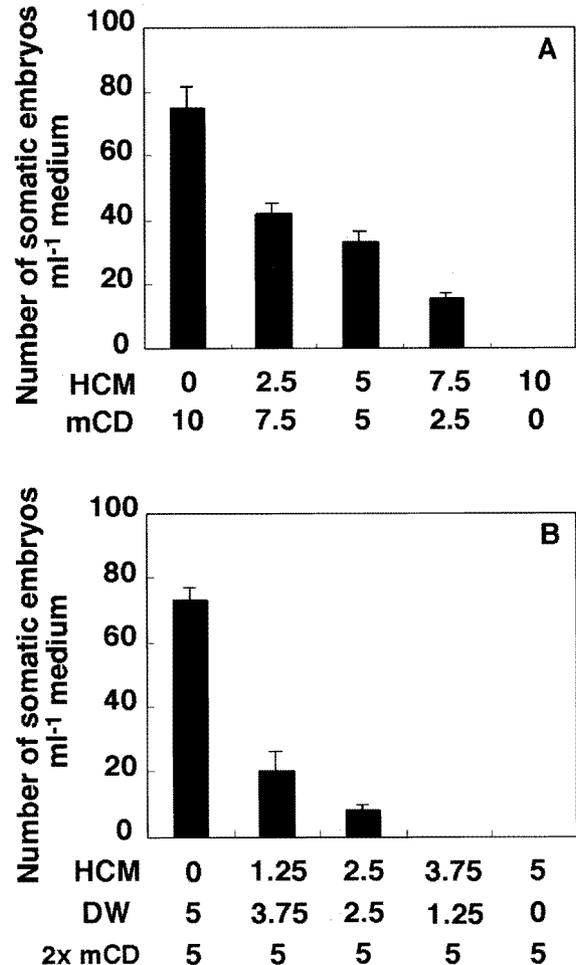
#### Effects of initial cell density on somatic embryogenesis

When the initial cell density was lower than  $0.1 \text{ ml PCV l}^{-1}$ , most EC did not proliferate, and only a few somatic embryos were formed (Fig. 1A-C, 2). This suggests that there is some threshold value of



**Fig. 2** Effect of initial cell density on somatic embryo formation. EC were suspended in phytohormone-free mCD medium at various cell densities and cultured for three weeks. The data are presented as the number of embryos formed per ml PCV at the start of culture. The bars indicate the standard error ( $n = 5$ ).

the cell density for the formation of somatic embryos in Japanese larch cells. When the initial cell density was  $0.5 \text{ ml PCV l}^{-1}$ , many somatic embryos (*ca.*  $2.7 \times 10^4 \text{ l}^{-1}$ ) consisting of a dense cytoplasmic embryo-proper and a vacuolated suspensor were formed three weeks after the culture (Fig. 1D, 2). The somatic embryos were allowed to mature in a solidified mCD medium containing  $3 \text{ g l}^{-1}$  Gellan gum and  $50 \mu\text{M}$  ABA (see Materials and Methods). They developed into plantlets four months after



**Fig. 3** Effect of the addition of HCM to mCD medium on somatic embryo formation. (A) Number of somatic embryos plotted against the amount of HCM added. Abscissa shows the amounts of HCM and mCD medium in 10 ml of each test medium. (B) Number of somatic embryos plotted against the amount of HCM added when the concentrations of mCD medium nutrients were equal to or higher than the initial concentrations. Abscissa shows the amounts of HCM, distilled water, and 2x mCD medium (whose mCD nutrients were two-fold strength) in 10 ml of each test medium. EC were suspended in each test medium at  $0.5 \text{ ml PCV l}^{-1}$  and cultured for three weeks. The bars indicate the standard error ( $n = 5$ ). See text for details.

transferred to phytohormone-free medium (Fig. 1G). On the other hand, the yield of somatic embryos was markedly decreased when the initial cell densities were greater than  $1.0 \text{ ml PCV l}^{-1}$  (Fig. 1E, F, 2). Most of the cells remained small and formed spherical cell clusters similar to EC (Fig. 1F), but did not differentiate into vacuolated suspensor-like structures. This feature was particularly pronounced at  $5.0 \text{ ml PCV l}^{-1}$ . When the cell clusters formed at  $5.0 \text{ ml PCV l}^{-1}$  were treated with  $50 \mu\text{M}$  ABA and transferred to phytohormone-free mCD medium, callus was formed and no somatic embryos or plantlets were formed (Fig. 1H). Taken together, it is concluded that the optimum cell density for inducing somatic embryos of Japanese larch is  $0.5 \text{ ml PCV l}^{-1}$ .

#### Effects of HCM

HCM strongly inhibited the formation of somatic embryos (Fig. 3A). The degree of inhibition increased with the volume of HCM added. This inhibitory effect of HCM suggests that a factor(s) inhibiting somatic embryogenesis was present in the HCM, or that the mCD nutrients were depleted by the addition of HCM, or both.

To investigate whether the inhibition was caused by nutrient depletion, the nutrients in each test medium were adjusted to be equal to or higher than those of the initial concentrations, as described in the *Materials and Methods*. If HCM contains insufficient nutrients, the supplement of nutrients to HCM can cause somatic embryogenesis. However, when HCM was added to mCD medium whose

nutrients were two-fold strength, the inhibition of somatic embryogenesis was stronger (Fig. 3B) than those when only HCM was added (Fig. 3A). These results indicate that HCM includes enough nutrients; the surplus nutrient in mCD medium may cause the strong inhibition. Therefore, inhibitory factor(s), which was secreted from suspension cells and accumulated in HCM, inhibited somatic embryogenesis.

#### Effects of activated charcoal on somatic embryo development

When activated charcoal was added to high-cell-density cultures ( $3.0$  and  $5.0 \text{ ml PCV l}^{-1}$ ), the number of somatic embryos was increased compared to cultures without activated charcoal (Fig. 4). Conversely, the addition of activated charcoal strongly inhibited somatic embryo formation in cultures at  $0.5 \text{ ml PCV l}^{-1}$  (Fig. 4). The effects of activated charcoal on the growth of somatic embryos (length of embryo-proper and suspensor) were examined (Fig. 5). Somatic embryos that formed in a medium containing activated charcoal had longer suspenders than those formed in a medium without activated charcoal at higher cell density of  $0.5 \text{ ml PCV l}^{-1}$  (Fig. 5A, B). The size of the embryo-proper did not differ in the media with or without activated charcoal (Fig. 5C, D, E).

#### Discussion

It is well known that a high-cell-density culture strongly inhibits somatic embryo formation in some plants, such as *Citrus sinensis* (Kobayashi *et al.*, 1985), *Brassica napus* (Grand *et al.*, 1988), and carrot (Halperin, 1967). Recently, it was clarified that this strong inhibition in carrot is caused by 4-hydroxybenzyl alcohol (4HBA), which is released from cultured cells and young somatic embryos (Kobayashi *et al.*, 2000a, b). 4HBA specifically inhibits rapid cell division that is characteristic of the early stage of globular embryo formation (Kobayashi *et al.*, 2001).

In order to examine the interaction between the embryo-proper and suspensor, we examined somatic embryogenesis in Japanese larch, in which the somatic embryos consist of an embryo-proper and suspensor. Somatic embryogenesis of Japanese larch was suppressed in high-cell-density culture. However, exogenously applied 4HBA did not inhibit somatic embryogenesis of Japanese larch (data not shown). We speculated that substances other than 4HBA may cause the inhibition of somatic embryogenesis. Furthermore, when the initial cell density exceeded  $1.0 \text{ ml PCV l}^{-1}$ , somatic embryo-

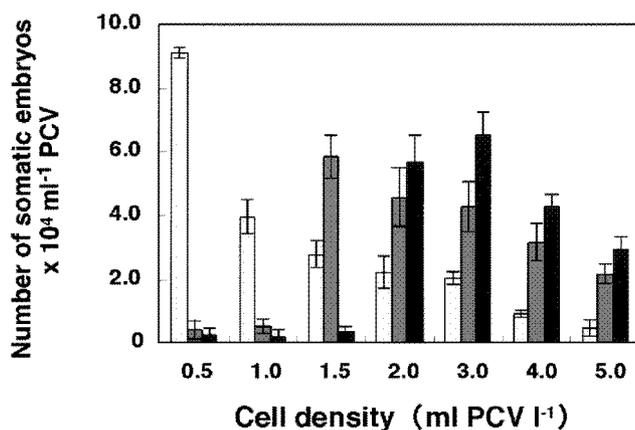
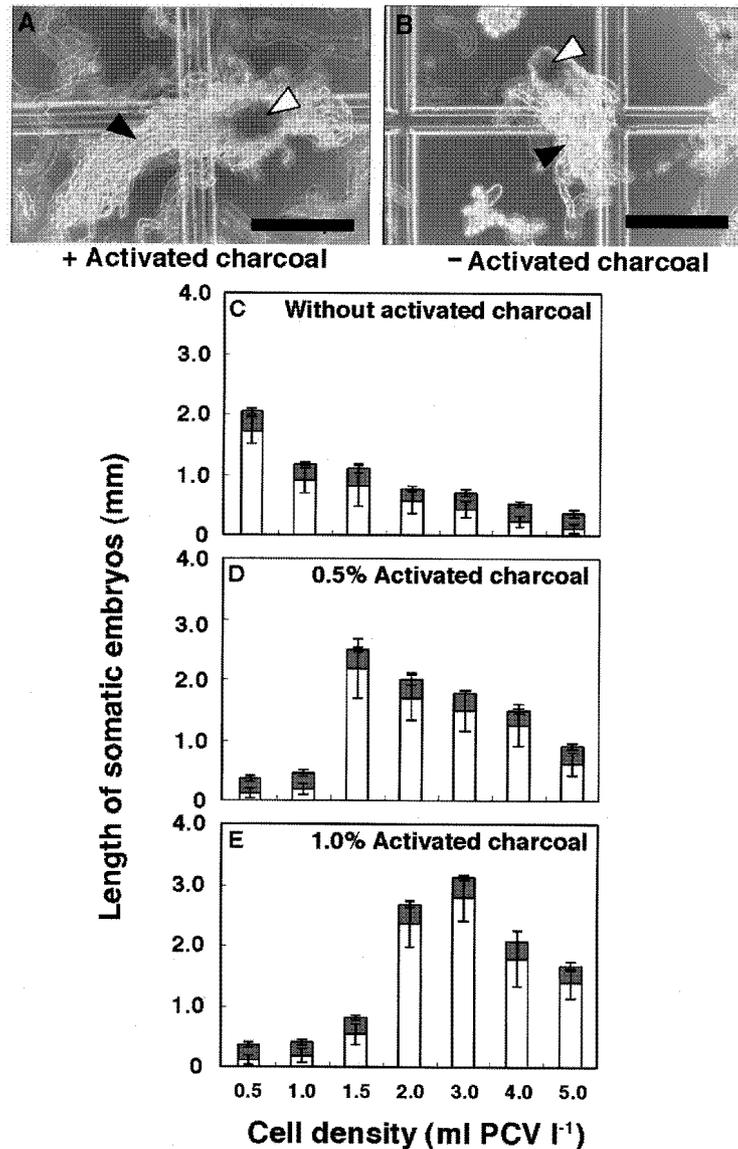


Fig. 4 Effect of activated charcoal on somatic embryo formation at various cell densities. EC were suspended in medium with 1% activated charcoal (black boxes), 0.5% activated charcoal (gray boxes), and without activated charcoal (white boxes) at  $0.5$ – $5.0 \text{ ml PCV l}^{-1}$  and cultured for three weeks. The bars indicate the standard error ( $n = 5$ ).



**Fig. 5** Effect of activated charcoal on the growth of somatic embryos. The culture conditions were the same as in Fig. 4. Somatic embryos formed in medium with (A) or without (B) activated charcoal. White arrowhead shows embryo-proper. Black arrowhead shows suspensor. The bar indicates 500  $\mu$ m. (C-E) Length of the embryos-proper (closed boxes) and suspensors (open boxes) cultured with or without activated charcoal were measured under a digital microscope. (C) Without activated charcoal, (D) 0.5% activated charcoal, (E) 1.0% activated charcoal. The bar indicates the standard error (n = 5).

genesis was strongly inhibited (Fig. 1, 2). At these cell densities, the suspensor was shorter, but the size of the embryo-proper was the same as that at low cell density (Fig. 1, 5C-E).

The inhibition of somatic embryogenesis with both HCM and nutrients was stronger than that with the addition of HCM alone (Fig. 3A, B), indicating that sufficient nutrients remained in HCM, and excess nutrients further inhibit somatic embryogenesis. These results suggest that the inhibitory effect of HCM was caused by an inhibitory factor(s) rather than nutrient depletion, which was released from the cells into the medium and caused the inhibition of somatic embryogenesis.

The addition of activated charcoal increased the

frequency of somatic embryo formation at high cell density (Fig. 4). This result supports the hypothesis that an inhibitory factor(s) released from the cells into the medium. Conversely, the number of somatic embryos at 0.5 ml PCV l<sup>-1</sup> was markedly reduced when compared to that without activated charcoal (Fig. 4). Factors that both stimulate and inhibit somatic embryogenesis have been found in carrot conditioned medium. The inhibitory factor was identified as 4HBA (Kobayashi *et al.*, 2000a, b, 2001), and the stimulatory factors were identified as phytosulphokine, and peptidyl plant growth factor (Kobayashi *et al.*, 1999b; Hanai *et al.*, 2000), and as arabinogalactan proteins in other experiments (van Hengel *et al.*, 2001). Therefore, the conditioned

medium of Japanese larch is thought to contain both factors that stimulate and inhibit somatic embryogenesis, and the amounts of each factor in the conditioned medium vary depending on cell density. At 0.5 ml PCV l<sup>-1</sup>, stimulatory factors may exceed inhibitory factor, and the addition of activated charcoal may absorb much more stimulatory factors than the inhibiting factor, which reduces the number of somatic embryos formed. It is supposed that the difference of effective concentration (threshold) of these factors caused the shift of optimum cell density between those with and without activated charcoal (Fig. 4). Furthermore, the somatic embryos that formed in cultures with activated charcoal had longer suspensors than those cultured without activated charcoal, and vacuolated cells-like suspensor elongated on the surroundings of the embryo-proper (Fig. 5A, B). Therefore, this inhibitory factor(s) might regulate elongation or development of the suspensor.

It appears that the inhibitory substance(s) and their physiological function differ between carrot and larch. The factor(s) inhibiting somatic embryogenesis in Japanese larch controls differentiation or development of the suspensor. In high-cell-density culture, suspensor elongation was strongly suppressed. It is likely that the suspensor also produces stimulatory factor(s) to stimulate development of the embryo-proper, and the balance in the production of these stimulatory and inhibitory factors controls embryo development. Currently, we are attempting to identify the chemicals that serve as inhibitory and stimulatory factors and to clarify details of the effects of the interaction of these factors on embryo differentiation and development.

### Acknowledgement

This work was supported in part by a Grant-in-Aid from the "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF00L01601), and by a Grant-in-Aid and special coordination funds from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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