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Inhibitory Effects of *p*-Hydroxybenzyl Alcohol on Somatic Embryogenesis in Carrot Cell Cultures

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Abstract

Somatic embryogenesis in carrot (*Daucus carota* L.) is strongly inhibited by certain factors that accumulate in the medium of cultures with high embryogenic carrot cell density. We previously identified *p*-hydroxybenzyl alcohol as one of these inhibitory factors. In this study, we performed a detailed analysis of the effects of *p*-hydroxybenzyl alcohol on somatic embryogenesis. *p*-Hydroxybenzyl alcohol strongly inhibited the formation of somatic embryos by suppressing cell division. The inhibitory effect on the development of globular embryos was stronger than that on the development of heart-shaped and torpedo-shaped embryos. In contrast, *p*-hydroxybenzyl alcohol had no effect on the proliferation of both undifferentiated embryogenic cells or non-embryogenic cells. These results indicate that *p*-hydroxybenzyl alcohol inhibits the division of cells in a way that is specific to the early stage of somatic embryogenesis and that plays an important role in the formation of somatic embryos.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; DW, distilled water; HCM, conditioned medium from high-cell-density culture; PCV, packed cell volume after centrifugation at 100 x*g*

1. Introduction

Various environmental and chemical factors influence the induction and development of carrot somatic embryos (Halperin, 1967; Ammirato and Steward, 1971; Kamada and Harada, 1979, 1984; LoSchiavo *et al.*, 1986). Auxins are the most important of these factors and the effects of the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), have been studied in considerable detail. Embryogenic cells, which have the ability to generate somatic embryos, can be obtained by culturing explants on 2,4-D-containing medium and transferring the resultant embryogenic cells to 2,4-D-free medium, in which somatic embryos are subsequently formed (Steward *et al.*, 1958; Reinert, 1959; Kamada and Harada, 1979).

Cell density also has important effects on carrot somatic embryogenesis (Fridborg *et al.*, 1979; Sung and Okimoto, 1981, 1983; Osuga *et al.*, 1993). When embryogenic cells are cultured in 2,4-D-free medium at high cell density, the formation of somatic embryos is strongly inhibited. Such inhibition is due to certain factors that are released into the culture medium from the cultured cells (Higashi *et al.*, 1998). The inhibitory factors appear to suppress only the exceptionally rapid division of cells that is characteristic of the early stages of somatic embryogenesis (Kobayashi *et al.*, 1999b). Such inhibitory endogenous factors were different from the natural and chemically synthesized factors that have been shown to inhibit the formation of somatic embryos (LoSciavo *et al.*, 1986; Baldan *et al.*, 1995; Capitano *et al.*, 1997; Toonen *et al.*, 1997). We identified one of the inhibitory endogenous factors as *p*-hydroxybenzyl alcohol (Kobayashi *et al.*, unpublished data), but the physiological actions of *p*-hydroxybenzyl alcohol remain to be characterized. In this study, we performed a detailed analysis of the effects of *p*-hydroxybenzyl alcohol on carrot cells in culture.

2. Materials and Methods

2.1 Plant material and cell culture

Details of the methods used for the culture of embryogenic cells and non-embryogenic cells of

Daucus carota L. cv. US-Harumakigosun have been described previously (Kamada and Harada, 1979; Satoh *et al.*, 1986). Embryogenic cells obtained from hypocotyls were subcultured at two-week intervals in liquid Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) that contained 2,4-D (1 mg l^{-1}). For induction of somatic embryogenesis, small cell clusters (37-63 μm in diameter) were collected by passing of two-week-old suspension cultures through two stainless-steel sieves (pore size, 37 and 63 μm, respectively). The clusters were washed with an excess of phytohormone-free MS medium and then suspended in phytohormone-free liquid MS medium at 0.2 m*l* PCV l^{-1}. Cell density was defined in terms of the packed cell volume (PCV), measured in m*l*, after centrifugation at 100 x*g* of one liter of culture (m*l* PCV l^{-1}).

Non-embryogenic cells were obtained by successive subculturing, at two-week intervals, of small clusters of cells of less than 1 mm in diameter for more than six months in liquid MS medium that contained 2,4-D (1 mg l^{-1}). Non-embryogenic cells aggregated loosely and formed small clusters of cells. The non-embryogenic cells were maintained by subculture at two-week intervals in liquid MS medium that contained 2,4-D (1 mg l^{-1}).

All cultures were incubated on a gyratory shaker (70 rpm) at 25 ℃ in darkness.

2.2 Chemicals

p-Hydroxybenzyl alcohol and its analogs, namely *o*- and *m*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and benzyl alcohol (extra pure grade), were obtained from Nacalai tesque, Inc. (Kyoto, Japan). Each compound was dissolved in distilled water (DW) and solutions were sterilized by filtration through a cellulose acetate membrane with 0.45-μm pores (DISMIC-25cs; ADVANTEC, Tokyo).

2.3 Effects of p-hydroxybenzyl alcohol on the formation of somatic embryos

Small clusters (37 to 63 μm in diameter) of embryogenic cells, collected as described above, were suspended at 0.2 m*l* PCV l^{-1} in phytohormone-free MS medium that contained *p*-hydroxybenzyl alcohol or an analog. After culture for two weeks, the number of somatic embryos at each stage of development in a 500-μl aliquot of culture was determined in a counting chamber under a microscope.

2.4 Stage specific effect of p-hydroxybenzyl alcohol on the formation of somatic embryos

After the embryogenic cells had been cultured for 14 days at an initial cell density of 0.2 m*l* PCV l^{-1}, globular, heart-shaped and torpedo-shaped somatic embryos were collected separately. The embryos at each stage of development were suspended in phytohormone-free MS medium that contained *p*-hydroxybenzyl alcohol and cultured for 14 days. Then, somatic embryos and cells were counted as described below.

To count the total cells in various cultures, all of cultured cells were collected by centrifugation at 100 x*g* for five minutes. The pelleted cells were treated for one day with an excess of maceration solution, which consisted of 10% (w/v) HNO_3 and 10% CrO_3. This mixture was then centrifuged at 100 x*g* and the pellet was washed once with DW. After centrifugation, the final pellet of cells was suspended in and diluted to an appropriate cell density with DW so that the cells could be counted in a hemocytometer (Thoma; Erma, Tokyo).

2.5 Effects of p-hydroxybenzyl alcohol on the proliferation of embryogenic cells and non-embryogenic cells

Small clusters of embryogenic cells (37-63 μm in diameter) and non-embryogenic cells were cultured at an initial cell density of 0.2 m*l* PCV l^{-1} in the medium that contained *p*-hydroxybenzyl alcohol with or without 2,4-D. Total cells were counted on the 14th day of culture as described in the previous section.

In all experiments, cultures were incubated in 50-m*l* flasks that contained 15 m*l* of test medium. In each experiment, at least four flasks were used. All experiments were repeated at least twice and average values of the results are shown with SD.

3. Results

3.1 Effects of p-hydroxybenzyl alcohol and its analogs on somatic embryogenesis

The number of somatic embryos formed after two weeks decreased significantly with increases in the concentration of *p*-hydroxybenzyl alcohol in the medium **(Fig. 1)**. However, somatic embryos at the heart-shaped and torpedo-shaped stages were generated even at a relatively high concentration (10^{-5} M). However, the proportion of somatic embryos at each developmental stage (globular, heart- and torpedo-shaped embryos) was not affected by addition of *p*-hydroxybenzyl alcohol. The number of somatic embryos was also reduced when analogs of *p*-hydroxybenzyl alcohol were included in the

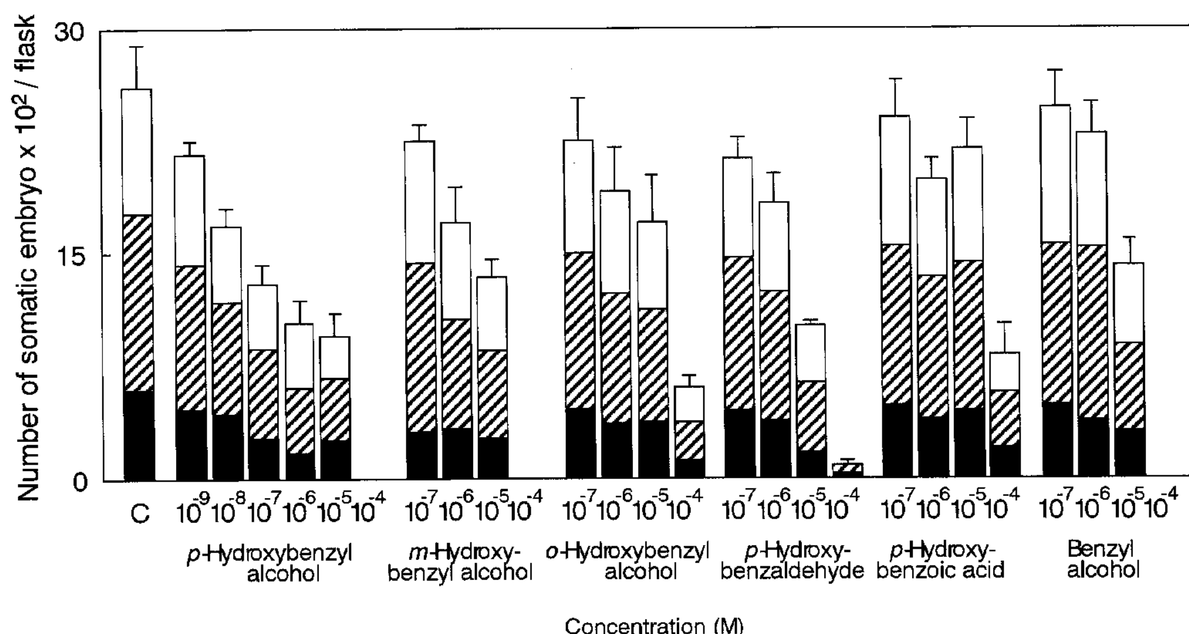

Fig. 1 Effects of p-hydroxybenzyl alcohol and its analogs on somatic embryogenesis. Small clusters of embryogenic cells were suspended at 0.2 m*l* PCV l^{-1} in the phytohormone-free MS medium that contained p-hydroxybenzyl alcohol or an analog. After 14 days of culture, somatic embryos at each stage of development were counted. Closed boxes, globular embryos; stripped boxes, heart-shaped embryos; open boxes, torpedo-shaped embryos. Results represent mean values with SD (n=4). No somatic embryos developed in the medium that contained p-, m-hydroxybenzyl alcohol, p-hydroxybenzoic acid or benzyl alcohol at 10^{-4} M of concentration.

medium. However, the inhibitory effects of the analogs that we tested were weak compared to those of p-hydroxybenzyl alcohol. The concentrations of individual analogs that significantly inhibited the formation of somatic embryos were 10- to 100-fold higher than those of p-hydroxybenzyl alcohol that had similar effects.

3.2 Stage-specific inhibition by p-hydroxybenzyl alcohol of the formation of somatic embryos and cell proliferation

When we used globular somatic embryos as starting material **(Fig. 2a)**, we found that the number of somatic embryos after two weeks was reduced by culture in medium that contained p-hydroxybenzyl alcohol. The extent of inhibition depended on the concentration of the reagent. When we used heart-shaped embryos **(Fig. 2b)**, the number of somatic embryos that developed after two weeks was reduced in the presence of p-hydroxybenzyl alcohol at 10^{-5} M. In contrast, p-hydroxybenzyl alcohol had no effect on the number and development of torpedo-shaped embryos after two weeks **(Fig. 2c)**. The effects of p-hydroxybenzyl alcohol on the proliferation of cells were similar to those on the formation of somatic embryos when somatic embryos at each developmental stage were used **(Fig. 2d-f)**. When globular embryos were cultured in the medium that contained p-hydroxybenzyl alcohol, the proliferation of cells was strongly suppressed. The total number of cells in cultures of torpedo-shaped embryos was more than that in cultures of globular and heart-shaped embryos, because torpedo-shaped embryos grew bigger. However, p-hydroxybenzyl alcohol did not affect the increases of total cell number in cultures of heart and torpedo-shaped embryos.

3.3 Effects of p-hydroxybenzyl alcohol on the proliferation of embryogenic and non-embryogenic cells

Embryogenic cells proliferated and grew into small callus without any apparent morphological changes in the presence of 2,4-D. Under these conditions, p-hydroxybenzyl alcohol had no effect on the proliferation of embryogenic cells **(Fig. 3a)**. In contrast, the increase in number of total cells of somatic embryos and cell clusters during somatic embryogenesis was strongly suppressed upon addition of p-hydroxybenzyl alcohol to 2,4-D-free medium **(Fig. 3b)**.

The clusters of non-embryogenic cells, which had lost embryogenic competence, were uniformally small and loosely aggregated. Non-embryogenic cells proliferated in the presence and in the absence of 2,4-D. p-Hydroxybenzyl alcohol had no

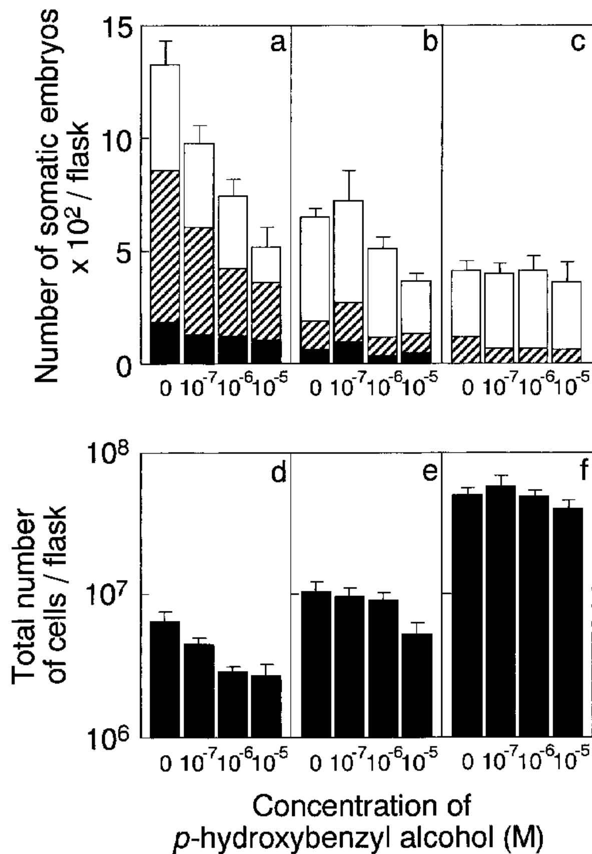

Fig. 2 Stage-specific effects of *p*-hydroxybenzyl alcohol on somatic embryogenesis. Globular (**a**, **d**), heart-shaped (**b**, **e**) and torpedo-shaped (**c**, **f**) embryos were separately suspended in medium that contained *p*-hydroxybenzyl alcohol. After 14 days of culture, the numbers of somatic embryos and cells were determined. **a**-**c**: The number of somatic embryos. Closed boxes, globular embryos; stripped boxes, heart-shaped embryos; open boxes, torpedo-shaped embryos. **d**-**f**: The total number of cells indicated by closed boxes. Results represent mean values with SD (n=4).

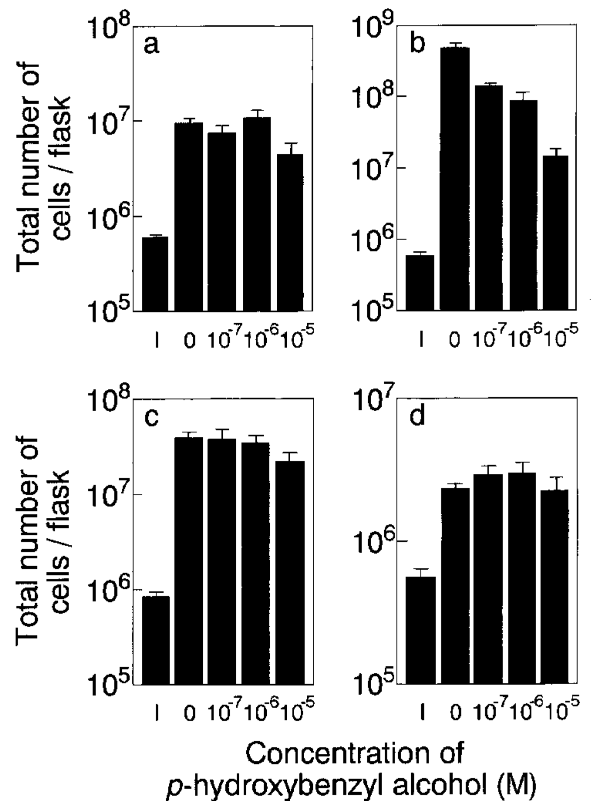

Fig. 3 Effects of *p*-hydroxybenzyl alcohol on the proliferation of embryogenic and non-embryogenic cells. Small clusters of embryogenic cells and of non-embryogenic cells were suspended at 0.2 m*l* PCV l^{-1} in medium that contained *p*-hydroxybenzyl alcohol with or without 2,4-D. After 14 days of culture, the total number of cells was determined. **a** and **b**: Embryogenic cells were cultured in medium with 2,4-D (**a**) and without 2,4-D (**b**). **c** and **d**: Non-embryogenic cells were cultured in medium with 2,4-D (**c**) and without 2,4-D (**d**). **I** indicates the total number of cells at the start of culture. Results represent mean values with SD (n=4 or 6).

effect on the proliferation of non-embryogenic cells in the presence and in the absence of 2,4-D (**Fig. 3c, d**).

4. Discussion

p-Hydroxybenzyl alcohol was identified previously as one of the inhibitory factors in conditioned medium that is responsible for the suppression of somatic embryogenesis in high-cell-density cultures (Kobayashi *et al.*, unpublished data). In this study, we examined in detail the effects of *p*-hydroxybenzyl alcohol and its analogs on somatic embryogenesis. *p*-Hydroxybenzyl alcohol inhibited the formation of somatic embryos more strongly than did the tested analogs (**Fig. 1**). It was demonstrated that the cell division during somatic embryogenesis was strongly inhibited in cultures at high cell density (Kobayashi *et al.*, 1999b) The strong inhibitory effect of *p*-hydroxybenzyl alcohol was also due to suppression of cell proliferation during somatic embryogenesis (**Fig. 3b**). The unusually rapid cell division occurs when small clusters of embryogenic cells develop into early globular embryos (Bayliss, 1977). This rapid cell division plays an important role in the initiation of somatic embryogenesis (Fujumura and Komamine, 1980). During the culture of globular somatic embryos, the number of somatic embryos was increased because of the formation of secondary somatic embryos (**Fig. 2a**). Some secondary somatic embryos were also formed in

cultures of heart- and torpedo-shaped embryos **(Fig. 2b, c)**. The secondary somatic embryos seemed to develop actively from somatic embryos at earlier stage of development. The inhibitory effect of *p*-hydroxybenzyl alcohol on the development of globular embryos was stronger than that on the development of heart-shaped and torpedo-shaped embryos **(Fig. 2)**. The unusually rapid division of cells occurs only during the development of somatic embryos. *p*-Hydroxybenzyl alcohol had no effect on the proliferation of undifferentiated embryogenic cells **(Fig. 3a)** and of non-embryogenic cells **(Fig. 3c, d)**. Thus, *p*-hydroxybenzyl alcohol inhibited only rapid division of cells of somatic embryos that is characteristic of the early globular stage of somatic embryogenesis and did not affect the proliferation of undifferentiated embryogenic cells and non-embryogenic cells. This physiological property of *p*-hydroxybenzyl alcohol is the same as that of the inhibitory conditioned medium isolated (Kobayashi *et al.*, unpublished data). Therefore, *p*-hydroxybenzyl alcohol appears to be the major inhibitory factor that accumulates in the medium of high-cell-density cultures and inhibits somatic embryogenesis.

Several phytohormones and chemicals are known to suppress somatic embryogenesis in carrot cell cultures. The existence and concentrations of inhibitors in conditioned medium have not previously been determined, except in the case of *p*-hydroxybenzoic acid. Fridborg *et al.* (1979) reported that *p*-hydroxybenzoic acid accumulates in the medium during carrot somatic embryogenesis and inhibits the formation of somatic embryos. However, we found that *p*-hydroxybenzoic acid had a much smaller inhibitory effect on somatic embryogenesis than that of *p*-hydroxybenzyl alcohol. In addition, we did not find any *p*-hydroxybenzoic acid in our high-cell-density cultures (Kobayashi *et al.*, unpublished data). Thus, it appears that *p*-hydroxybenzoic acid is not essential for the inhibition of somatic embryogenesis in high-cell-density cultures. Certain other chemicals specifically interrupt the transition from heart-shaped embryos to torpedo-shaped embryos (LoSchiavo *et al.*, 1986; Baldan *et al.*, 1995; Capitano *et al.*, 1997). In contrast, *p*-hydroxybenzyl alcohol markedly reduced the number of somatic embryos but a few somatic embryos develop into mature embryos **(Fig. 1)**. Such inhibitory effects of *p*-hydroxybenzyl alcohol might have been due to suppression of quite an early process in somatic embryogenesis. As a consequence, addition of *p*-hydroxybenzyl alcohol reduced the frequency of formation of somatic embryos and did not affect the proportion of somatic embryos at each developmental stage (globular, heart- and torpedo-shaped embryos).

Some factors that stimulate carrot somatic embryogenesis are also present in the conditioned medium. For example, arabinogalactan proteins (Kreuger and van Horst, 1993; Toonen *et al.*, 1997) and phytosulfokine-α (Matsubayashi *et al.*, personal communication; Kobayashi *et al.*, 1999a). Thus, somatic embryogenesis in carrot is probably influenced by a balance between levels of stimulatory and inhibitory conditioning factors. However, the inhibitory effects of *p*-hydroxybenzyl alcohol seem to overwhelm the effects of the stimulatory factors since *p*-hydroxybenzyl alcohol prevented development of somatic embryos at the early globular stage. We are now examining in further detail the accumulation of the stimulatory and inhibitory factors in the conditioned medium of carrot cultured cells.

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References

Ammirato, P.V., Steward, F.C., 1971. Some effects of environment on the development of embryos from cultured free cells. Bot. Gaz., **132:** 149-158.

Baldan, B., Frattini, C., Guzzo, F., Branca, C., Terzi, M., Mariani, P., LoSchiavo, F., 1995. A stage-specific block is produced in carrot somatic embryos by 1,2-benzisoxazole-3-acetic acid. Plant Sci., **108:** 85-92.

Bayliss, M.W., 1977. The effects of 2,4-D on growth and mitosis in suspension culture of *Daucus carota*. Plant Sci. Lett., **8:** 99-103.

Capitano, G., Baldan, B., Filippini, F., Terzi, M., LoSchiavo, F., Mariani, P., 1997. Morphogenetic effects of Brefeldin A on embryogenic cell cultures of *Daucus carota* L. Planta., **203:** 121-128.

Fridborg, G., Pedersen, M., Landstorm, L., Eriksson, T., 1978. The effect of activated charcoal on tissue cultures; adsorption of metabolites inhibiting morphogenesis. Physiol. Plant., **43:** 104-106.

Fujumura, T., Komamine, A., 1980. The serial observation of embryogenesis in a carrot cell suspension culture. New Phytol., **86:** 213-218.

Halperin, W., 1967. Population density effects on embryogenesis in carrot-cell cultures. Exp. Cell Res., **48:** 170-173.

Higashi, K., Daita, M., Kobayashi, T., Sasaki, K., Harada, H., Kamada, H., 1998. Inhibitory conditioning for carrot

somatic embryogenesis in high cell density cultures. Plant Cell Rep., **18:** 2-6.

Kamada, H., Harada, H., 1979. Studies on the organogenesis in carrot tissue cultures. II. Effects of amino acids and inorganic nitrogenous compounds on somatic embryogenesis. Z. Pflanzenphysiol., **91:** 453-463.

Kobayashi, T., Eun, C-H., Hanai, H., Matsubayashi, Y., Sakagami, Y., Kamada, H., 1999a. Phytosulfokine-?, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. J. Exp. Bot., **50:** 1123-1128.

Kobayashi, T., Higashi, K., Saitou, T., Kamada, H., 1999b. Physiological properties of inhibitory conditioning factor(s), inhibitory to somatic embryogenesis, in high-cell-density cultures of carrot. Plant Sci., **144:** 69-75.

Kreuger, M., van Horst, G.J., 1993. Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta., **189:** 234-248.

LoSciavo, F., Quesada-Allue, L.A., Sung, Z.R., 1986. Tunicamycin affects somatic embryogenesis but not cell proliferation of carrot. Plant Sci., **44:** 65-71.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15:** 473-497.

Osuga, K., Kamada, H., Komamine, A., 1993. Cell density is an important factor for synchronization of the late stage of somatic embryogenesis at high frequency. Plant Tissue Culture Lett., **10:** 180-183.

Reinert, J., 1959. Uber die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. Planta., **53:** 318-333.

Satoh, S., Kamada, H., Harada, H., Fujii, T., 1986. Auxin-controlled glycoprotein release into the medium of embryogenic carrot cells. Plant Physiol., **81:** 931-933.

Steward, F.C., Mapes, M.O., Mears, K., 1958. Growth and organized development of cultured Cells. I Growth and division of freely suspended Cells. Amer. J. Bot., **45:** 693-703.

Sung, Z.R., Okimoto, R., 1981. Embryogenic proteins in somatic embryos of carrot. Proc. Natl. Acad. Sci. USA, **78:** 3683-3687.

Sung, Z.R., Okimoto, R., 1983. Coordinating gene expression during somatic embryogenesis in carrots. Proc. Natl. Acad. Sci. USA, **80:** 2661-2665.

Toonen, M.A.J., Schmidt, E.D.L., van Kammen, A., de Vries, S.C., 1997. Promotive and inhibitory effects of diversed arabinogalactan proteins on *Daucus carota* L. somatic embryogenesis. Planta., **203:** 188-195.