DENSITY OF SUSPENSION CULTURE OF SOMATIC EMBRYOGENIC CELLS OF *LARIX LEPTOLEPIS* DETERMINES THE FATE OF DEVELOPMENT: INVOLVEMENT OF PHOSPHATE UPTAKE AND METABOLISM

Hiroshi Ashihara[a]*, Hamako Sasamoto[b] and Shinjiro Ogita[c]

Keywords: *Larix leptolepis;* Pinaceae; Japanese larch; cell culture; somatic embryogenesis; cell density; phosphate; nucleic acid; phospholipid.

Cell density of suspension culture of *Larix leptolepis* embryogenic cells determined the fate of the development. In a low-density cell culture, somatic embryo development was promoted, but the cells continued to proliferate and formed small spherical cell aggregates in a high-density cell culture. In the present study, we compared the uptake and metabolism of ^{32}P-labelled inorganic phosphate (P_i) by the cells of low- (1 %) and high (10 %)-density cultures during first 30 h. Little difference was found in the uptake of ^{32} P_i by the cells in both the low- and high density-cultures up to 6 h, but Pi uptake in high-density culture almost ceased at 6 hr after inoculation. In contrast, P_i uptake continued linearly in the low-density culture. ^{32}P was distributed in small molecular organic compounds (mainly nucleotides and sugar phosphates), nucleic acids, phospholipids, and phosphoproteins along with unmetabolized Pi. The incorporation of ^{32}P into nucleotide and phospholipid fractions was significantly higher in the cells of low-density culture at 30 h after inoculation. No marked difference on the endogenous levels of purine and pyrimidine nucleotides was observed in both density cultures. From these results it is speculated that the *de novo* nucleic acids and phospholipids synthesis required in the conversion of embryonic cells into somatic embryos may be caused by sufficient amounts of P_i from the medium occurring only in cells of the low-density culture. The nucleotide levels in both cultures were similar at least up to 72 h. Therefore, sufficient levels of nucleotides for proliferation may be supplied even in cells of the high-density culture. The involvement of P_i uptake in the phenomena observed in the low- and high-density cultures is discussed.

* Corresponding Author
Fax: +81-3-5700-4225
E-Mail: ashihara.hiroshi@ocha.ac.jp
[a] Department of Biology, Ochanomizu University, Tokyo, 112-8610, Japan
[b] Research Institute for Integrated Science, Kanagawa University, Hiratsuka, Kanagawa, 259-1293, Japan
[c] Biotechnology Research Center, Toyama Prefectural University, Imizu, Toyama,939-0398, Japan

Introduction

Japanese larch (*Larix leptolepis*) is an important tree in forestry plantations in Japan. The optimization of tissue culture methods has allowed the generation and propagation of viable plants from somatic cells in culture through somatic embryogenesis. Somatic embryogenesis results in the generation of bipolar structures with a well defined root-shoot axis. The techniques have been successfully employed in *L. leptolepis*, an economically important species in Japan, where it is utilized by the forestry industry for wood and lumber production.[1] However, in contrast to other woody plant species such as *Picea glauca*,[2] *L. leptolepis* culture has not been used for physiological and biochemical studies to reveal the mechanism on embryogenesis.

The morphogenetic changes of embryogenic cells which are caused by different cell densities in suspension culture of some Japanese conifers have been reported by Ogita et al.[1,3] They found that in a low cell density culture of *L. leptolepis*, development of embryogenic tissue into somatic embryos was promoted, while in a high cell density culture, the embryogenic tissue continued to proliferate as small spherical cell aggregates and no further development occurred (Fig. 1). The aim of this study is to elucidate the causes of the changing mechanism from embryonic cells to somatic embryos biochemically.

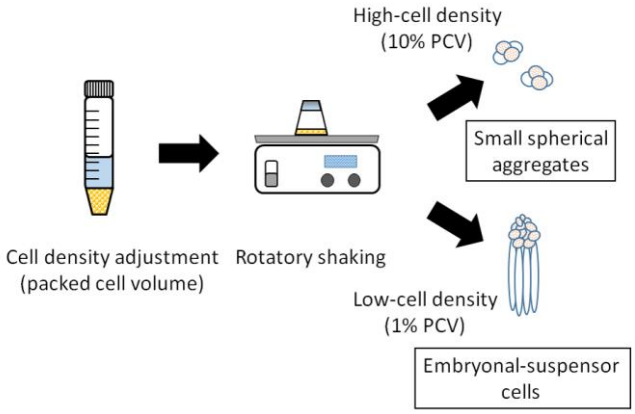

Figure 1. Outline of the high- and low-density cell suspension culture of *Larix leptolepis*. The somatic embryos are produced only in low-density culture.

The growth and development of cultured plant cells were largely influenced by the utilization of nutrients of culture medium. Among the many inorganic nutrients, inorganic phosphate (P_i) has been shown to participate in many metabolic processes affecting the growth and development.[4,5]

Overall changes in cellular metabolism by different concentration of P_i have been observed. In the cells grown in low availability of P_i, several metabolic changes were observed, including reduced rates of respiration,[6,7] decreased protein and nucleic acid synthesis,[8,9] development of P_i - deficient inducible bypasses of respiration,[7,10,11] as well as changes in nucleotide pool size and composition.[6,12-15] The growth of culture is strictly dependent upon P_i availability in the medium. Complete uptake of P_i by cultured plant cells is generally achieved during the first few days after inoculation into fresh medium.[16,17]

In the present studies, we tested the hypothesis that the supply of P_i to individual cells of *L. leptolepis* is satisfactory in a low-density culture, but not in a high-density culture where supply of P_i is limited. As a result of these differences, the embryogenic cells continue to proliferate as small spherical cell aggregates and no further development occurred. In order to elucidate the involvement of P_i uptake in the different developing pattern in the low- and high-density cultures, uptake and metabolism of $[^{32}P]P_i$ were compared. Furthermore, the cellular level of nucleotides was also determined.

Materials and Methods

Plant materials

Embryogenic tissues of *L. leptolepis* (=*L. kaempferi*) were maintained on the modified Campbell and Durzan (mCD, 1975) medium[18] which contained 7 µM 2,4-dichlorophenoxyacetic acid, 3 µM 6-benzylaminopurine, half a concentration of NH_4NO_3 (400 mg L^{-1}) and an additional 600 mg L^{-1} of L-glutamine. Liquid suspensions of the embryogenic tissues were generated as shown in the previous paper.[1] In order to investigate the effects of different cell densities on the proliferation and development of embryogenic cells, different volumes of cells (1 and 10 % as packed cell volume) were added to a 10 ml liquid mCD medium. The flasks were then placed on a rotary shaker with a speed of 100 rpm. The packed cell volume was determined by placing embryogenic cells in conical tubes and centrifuged using a centrifuge (Model 5400, Kubota Corporation, Tokyo, Japan) at 500 rpm for 3 min.

^{32}P-tracer experiments

The tracer experiments performed in this study are essentially the same as described in our earlier paper.[19] Sodium dihydrogen $[^{32}P]$orthophosphate (specific activity 7.4 GBq $mmol^{-1}$) were obtained from Amersham International plc, Amersham, UK. Experimental cultures were initiated from 10-day-old stock cultures at low- and high- inoculum density; 1 % and 10 % cultures were obtained by the transfer of 100 mg FW cells into 10 ml and 1 ml culture media in 50 ml- and 10 mL-conical flask flasks, respectively. The culture medium contained 1.25 mM $[^{32}P]KH_2PO_4$ (specific activity, 3.7 MBq mL^{-1}). The cultures were grown in the dark at 27 °C on a horizontal rotary shaker. The amount of P_i added as $[^{32}P]P_i$ is negligible in comparison to the original concentration in the medium.

The total cells of each flask were harvested by vacuum filtration through a layer of Miracloth (Calbiochem-Behring, La Jolla, U.S.A.) and washed with 100 ml distilled water. The fresh weight of the cells was determined after the washed cells had been left between four layers of filter paper to absorb water. The cells were dried overnight at 80 ºC. Total radioactivity was then measured using a Geiger-Muller counter connected to a digital recorder. Correction for radioactive decay was made by direct comparison of sample count rates with a standard containing $[^{32}P]P_i$.

The analysis of ^{32}P-metabolites was carried out by the modified Ashihara and Tokoro method.[19] A flow sheet of the separation of the labelled metabolites is shown in Fig. 2. The cells (~100 mg) harvested and washed as described above were homogenized in 4 ml of 6 % perchloric acid (PCA) for 2 min in an ice bath using a Potter-Elvehjem homogenizer. The homogenate was extracted successively with (i) 6 % PCA at 2 °C for 20 min (twice), (ii) an ethanol-ether (1: 1 v/v) mixture at 50 °C for 15 min (twice), (iii) 6 % PCA at 100 °C for 15 min and at 2 °C for 15 min, (iv) 3 N NaOH at 100 °C for 20 min. The first PCA-soluble fraction was neutralized with KOH, and after removal of the precipitated potassium perchlorate, a portion (4 ml) was concentrated *in vacuo*, and was analysed by thin-layer chromatography (TLC) using microcrystalline cellulose plates. The solvent system used was *n*-butanol-acetic acid-water (4:1:2 v/v).[20] Most of the radioactivity in the PCA soluble fraction was recovered as Pi, nucleotides and sugar phosphates (data not shown). In this paper, distribution of ^{32}P in P_i and organic phosphates (the sum of the various nucleotides and sugars) is shown. In preliminary studies, the ethanol-ether soluble fraction was also analysed by TLC as shown in our previous paper[19] and confirmed that most radioactivity in this fraction was distributed in phospholipids including phosphatidylcholine (data not shown).

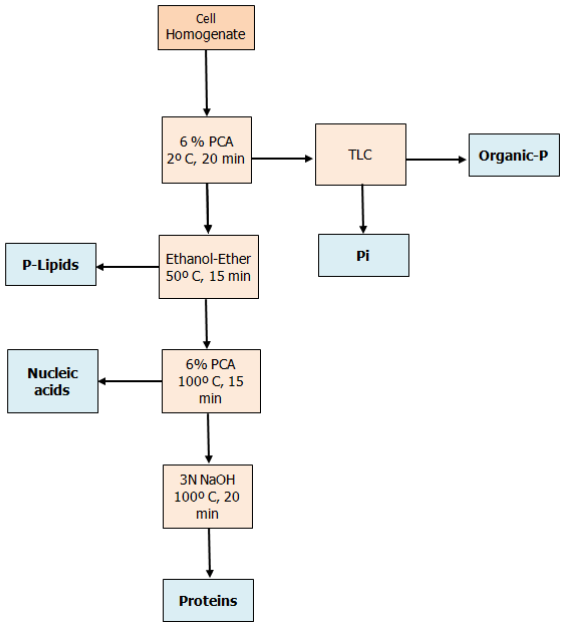

Figure 2. Flow sheet of the separation of ^{32}P-labelled metabolites.

The radioactivity in liquid fractions was determined using and scintillation fluid ACS-II (Amersham International plc, Amersham, UK) and a multi-purpose scintillation counter (type LS 6500, Beckman Instruments, Fullerton, CA, USA). Radioactivity on the TLC sheet was determined using a Bio-Imaging Analyser (Types FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan).

Determination of nucleotide level

Nucleotides were extracted from fresh cells (500 mg fresh weight) and were analysed using a HPLC system, as detailed in Ashihara et al.[21] with slight modifications. Nucleotide contents were determined using an anion exchange column, Shim-pack WAX-1 (Shimadzu Corporation, Kyoto, Japan). Freshly harvested cells were homogenized in chilled 6 % PCA with a glass homogeniser. The homogenates were centrifuged at 30,000 g for 15 min at 2 ºC, and the supernatant was collected and neutralized with 20 % KOH. After brief centrifuging to remove potassium perchlorate, the samples were lyophilised. The dried samples were dissolved in the solvent for HPLC and were filtered using disposable syringe filter units. Aliquots of 10–50 µl were used for determination by HPLC with a Shimadzu LC 10A HPLC system. The absorbance at 260 nm was monitored using a Shimadzu type SPD-10A, UV–Vis detector. Experiments to assess recovery were performed in parallel with all assays. Known amounts of standard were added to the extraction medium for one member of each pair of duplicate samples prior to homogenisation, and recovery was examined. Recoveries of standards were usually more than 90 %. Since some loss of nucleoside tri- and diphosphates was observed when the cells were frozen with liquid nitrogen and stored in a deep-freezer at -80 ºC, all assays were therefore performed using freshly harvested cells and were completed on the same day.

Results and Discussion

Cell density is an important factor that decides the fate of cultured cells.[1] In *L. leptolepis* cultures, low cell densities favour further development of the embryogenic tissue into embryonal-suspensor cell masses, while high densities result in the formation of small spherical cell aggregates. Morphology of embryogenic tissue after 4 weeks of culture in *L leptolepis* indicated that small spherical aggregates proliferated in a high-density culture (Fig. 3A). In contrast, an embryogenic tissue having a well organized embryonal head region and an elongated suspensor region formed in a low-density culture (Fig. 3B). In high-density cultures, rapid utilization of medium components might have resulted in some components becoming limiting factors for further growth and development. Since it has been reported that P_i is one of the important components that regulate the growth of plant cultures, we considered that P_i concentration might be closely related to the embryogenic tissue morphogenesis.

The total uptake of [^{32}P]P_i from the medium by the cells is shown in Fig. 4. P_i taken up by the cells was very rapid during first 3 hr in both high- and low-density cultures. The rate reached a plateau at 6 hr and then increased again. This rapid uptake was also found in suspension culture of *Catharanthus roseus*.[19] The large difference in the total uptake by the cells was observed in the high- and the low-density cultures at 30 hr after inoculation. The amounts of P_i uptake expressed per 100 mg fresh weight are 438 and 285 nmol in the low- and high-density cultures, respectively.

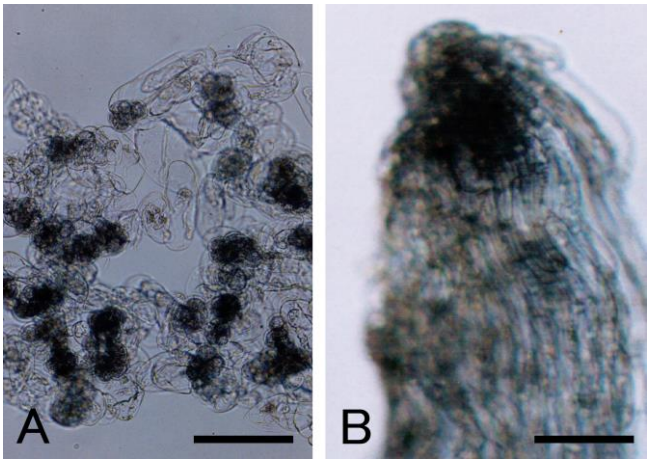

Figure 3. Morphology of embryogenic tissue after 4 weeks of culture in *L. leptolepis*. (A) Small spherical aggregates proliferated in the high-density culture. (B) An embryo having a well organized embryonal head region and an elongated suspensor region formed in a low-density culture. Scale bars represent 100 µm.

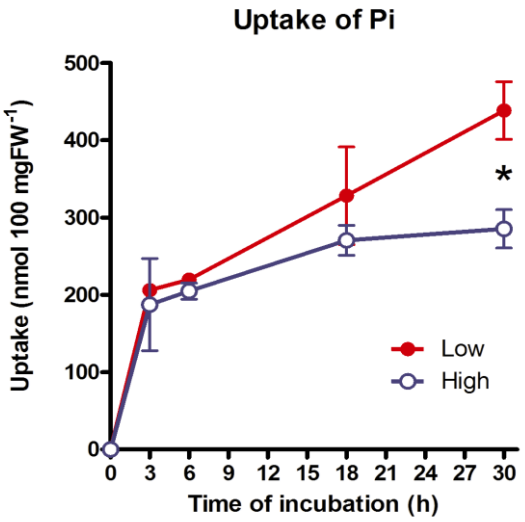

Figure 4. Uptake of [^{32}P]P_i by *L. leptolepis* cells cultured in the low- and high-density culture. The values are expressed as nmol 100 mg FW^{-1}. Values for high-density culture marked with asterisks differ significantly from the value for low-density culture according to the t-test ($P < 0.05$).

The incorporation of ^{32}P into individual cellular components is shown in Fig. 5. At 3 hr, nearly 70 % of the ^{32}P taken up by the cells was incorporated into organic components; the fractions of the PCA-soluble organic compounds consisted of nucleotides and sugar phosphates (50–52 %), nucleic acids (~9 %) and phospholipids (~6 %) and proteins (~0.5 %) in both low- and high-density cultures. Unmetabolized P_i was 32–35 % of total ^{32}P taken up by the cells. At 6 hr, distribution of radioactivity in nucleic acids (11–12 %) and phospholipid (10–12 %) was slightly increased. Nevertheless, small differences in the ^{32}P distribution into individual metabolites between two different density cultures were found up to 6 hr after inoculation.

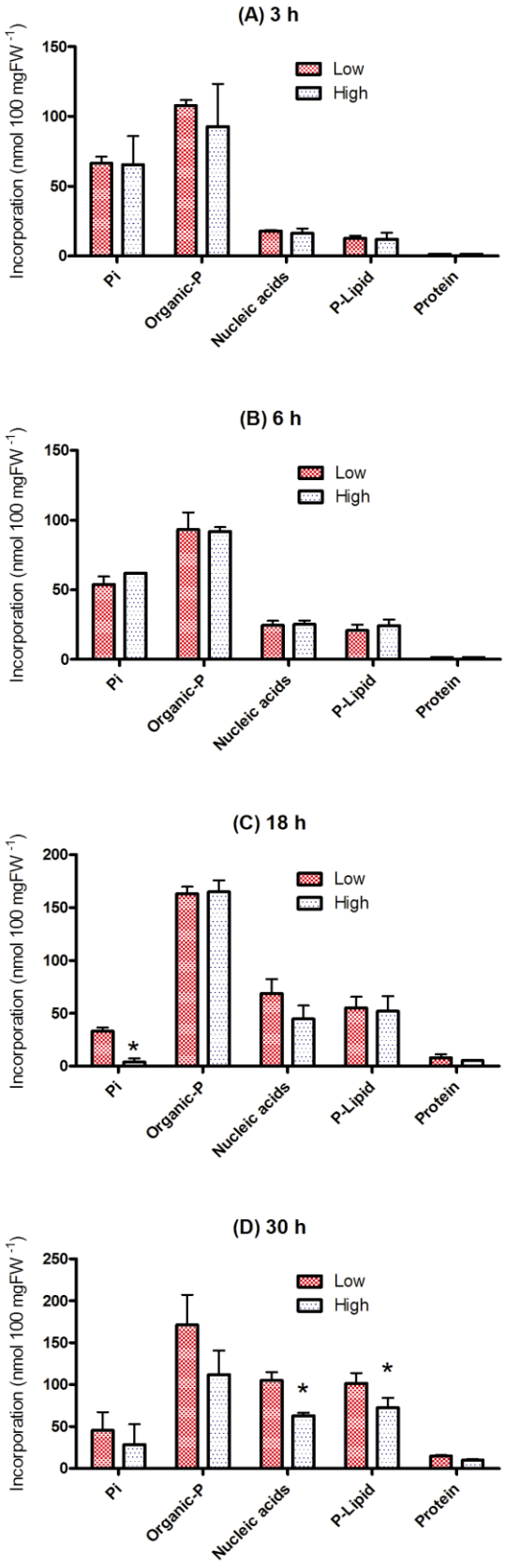

Figure 5. Distribution of ^{32}P taken up by *L. leptolepis* cells cultured in the low- and high-density culture. The samples are obtained at 3, 6, 18 and 30 h after inoculation. The values are expressed as nmol 100 mg FW^{-1}. Values for high-density culture marked with asterisks differ significantly from the value for low-density culture according to the *t*-test ($P < 0.05$). **P_i**, inorganic phosphate; **Organic-P**, cold PCA-soluble ^{32}P metabolites consists of nucleotide and sugar phosphates; **Nucleic acids**, RNA and DNA; **P-Lipid**, phospholipids; **Protein**, phosphoproteins.

The cellular [^{32}P]P_i level, especially in the high density-cultures, decreased 18 hr after inoculation (1–10 % of total ^{32}P taken up by the cells), and more than 90 % of ^{32}P was incorporated into the organic components. Although little difference in incorporation into nucleotides, sugar phosphates, phospholipids and phosphoproteins was found between the low- and high-density cultures, amounts of incorporation into nucleic acid in the low density culture (68.8 nmol) was higher in the high- density cultures (44.7 nmol). The trend was marked at 30 hr after culture where incorporation of ^{32}P into nucleic acid (105.2 nmol) and phospholipid (101.5 nmol) in the low density-cultures was higher than into those components (respectively, 62.8 and 72.3 nmol) in the high density-cultures.

In relation of phosphate and cell proliferation, Amino et al.[22] shows a linear relationship between initial P_i concentration in the medium (0–2.5 mM) and the stationary phase cell number, 10 days after inoculation. No cell number increase was observed in phosphate-free medium. These results indicate that P_i was limiting division of *Catharanthus roseus* cells. Ashihara et al.[23] determined the levels of purine and pyrimidine nucleotides in suspension cultures of *Catharanthus roseus* 24 h after stationary phase cells were transferred to fresh complete or phosphate-deficient medium containing 1.25 mM P_i. The levels of ATP, GTP, UTP and CTP were from approx. 3 to 5-fold greater in the cells grown in complete (with optimal level of P_i) medium than in the cells grown in the P_i -deficient medium. The levels of almost all other nucleotides were slightly higher in the cells in the complete medium. The authors suggested that increments in the levels of nucleotides, especially nucleoside triphosphates caused by P_i may induce a variety of biosynthetic reactions, such as nucleic acids, proteins, lipids and polysaccharides, and the proliferation of cells is believed to be triggered by such biochemical reactions.

To examine if increased level of nucleotides occurred in cells of the low-density culture, we extracted cellular nucleotides and analysed by HPLC. Table 1 shows the nucleotide levels in the cells grown in low density- and high-density cultures. All purine and pyrimidine nucleoside mono-, di- and triphosphates, except CMP were determined. The peak of CMP was sometimes contaminated by small amounts of impurities. No marked differences were found in nucleotide profiles in cells of the low- and high-density cultures. ATP levels in both cultures (~100 nmol gFW^{-1}) is similar to the value obtained from *C. roseus* cells in complete medium.[23] Compared to several cultured plant cells,[4] AMP level was high in *L. leptolepis* cells, as a result, the energy charge in the cells in the low- and high-density cultures varied 0.38–0.68 and 0.38–0.63, respectively. These values were much lower than those of plant cells reported,[4,24] although similar lower value was also found in *Datura innoxia* cells.[25] The results obtained here suggest that *L. leptolepis* cells even cultured in high-density culture possessed the sufficient amounts of nucleotides to continue proliferation at least up to 72 h.

For the formation of somatic embryos, induction of new RNA expression and rapid DNA replication seems to be required.[26] Therefore, sufficient P_i supply seems to be essential for the somatic embryogenesis of *L. leptolepis*. In general, embryogenic cells tissue is maintained in the presence of plant growth regulators, auxin and cytokinin.

Table 1. The effect of embryonic cell density on the concentration of nucleotides in *Larix leptolepis* cultures

	0 h	6 h		24 h		48 h		72 h	
		Low	High	Low	High	Low	High	Low	High
UMP	8±5	13±5	31±10	7±8	21±4	30±13	18±8	21±18	31±8
CMP	*	*	19±16	27±18	*	*	*	*	*
AMP	61±35	98±29	180±27	54±47	134±29	190±53	87±8	146±44	194±47
GMP	11±2	15±5	27±5	13±3	19±5	28±8	14±6	19±7	28±6
UDP	43±22	63±17	70±2	63±10	69±11	73±12	46±25	71±10	79±8
CDP	6±2	6±3	3±2	2±2	3±1	5±1	2±1	4±1	5±2
ADP	47±22	68±11	103±8	66±7	92±21	103±17	55±27	86±6	120±12
GDP	11±3	13±3	20±2	12±5	16±2	21±5	14±4	16±2	22±1
UTP	72±5	76±22	82±9	74±1	98±22	86±16	78±13	90±29	74±16
CTP	47±11	33±8	27±3	47±13	32±3	27±5	44±12	24±4	17±3
ATP	96±7	81±11	91±10	107±7	102±4	100±14	116±6	105±8	95±13
GTP	37±7	25±6	29±2	42±10	32±1	31±7	43±6	35±6	30±4

Nucleotide concentration is expressed as nmol gFW^{-1}. * not determined due to difficulty of the removal of contaminants.

Initiation of embryo development in suspension culture is induced by removal of plant growth regulators.[27] However, the present culture contained phytohormons throughout its entire culture period including induction of embryogenesis. Therefore, the effect of different amounts of plant growth regulators remained in the inoculated cultures as contaminants on the embryogenesis in low- and high-density cultures can be excluded.

In conclusion, the development of embryogenic cells into somatic embryos in the low-density culture seems to be complete supply of P_i, which is required for the synthesis of nucleic acid and phospholipid. In contrast, inhibition of the formation of somatic embryos in high-density culture is due to the limitation of P_i supply. Although other factors may be participated, difference in the phosphate availability for growth appeared to be one of the most important factors to determine the fate of development of cells of different density cultures.

Acknowledgements

The authors thank Professor Claudio Stasolla, University of Manitoba, and Professor Tatsuhito Fujimura, University of Tsukuba, for their valuable comments.

References

[1]Ogita, S., Sasamoto, H. and Kubo, T., *Transplant Production in the 21st Century*, Kluwer Academic Publishers, **2000,** 209-214.

[2]Stasolla, C. and Yeung, E. C., *Plant Cell Tiss. Org. Cult.*, **2003,** *74*, 15-35.

[3]Ogita, S., Kubo, T. and Fushitani, M., *Forest Resour. Environ.*, **1997**, *35*, 45-51.

[4]Wagner, K. G. and Backer, A. I., *Int. Rev. Cytol.*, **1992,** *134*, 1-84.

[5]Ukaji, T. and Ashihara, H., *Z. Naturforsch.*, **1986**, *41c*, 1045-1051.

[6]Li, X.N. and Ashihara, H., *Phytochemistry*, **1990**, *29*, 497-500.

[7]Nagano, M. and Ashihara, H., *Plant Cell Physiol.*, **1993**, *34*, 1219-1228.

[8]Ukaji, T. and Ashihara, H., *Ann. Bot.*, **1987**, *60*, 109-114.

[9]Li, X. N. and Ashihara, H., *Ann. Bot.*, **1989**, *64*, 33-36.

[10]Duff, S. M. G., Moorhead, G. B. G., Lefebvre, D. D. and Plaxton, W. C., *Plant Physiol.*, **1989**, *90*, 1275-1278.

[11]Nagano, M., Hachiya, A. and Ashihara, H., *Z. Naturforsch.*, **1994**, *49c*, 742-750.

[12]Ukaji, T. and Ashihara, H., *Int. J. Biochem.*, **1987**, *19*, 1127-1131.

[13]Dancer. J., Veith, R., Feil, R., Komor, E. and Stitt, M., *Plant Sci.*, **1990**, *66*, 59-63.

[14]Shimano, F. and Ashihara, H., *Phytochemistry*, **2006**, *67*, 132-141.

[15]Yin, Y., Shimano, F. and Ashihara, H., *J. Exp. Bot.*, **2007**, *58*, 1025-1033.

[16]Wylegalla, C., Meyer, R. and Wagner, K. G., *Planta*, **1985**, *166*, 446-451.

[17]Ashihara, H. and Ukaji, T., *J. Plant Physiol.*, **1986**, *124*, 77-85.

[18]Campbell, R. A. and Durzan, D. J., *Can. J. Bot.*, **1975**, *53*, 1652-1657.

[19]Ashihara, H. and Tokoro, T., *J. Plant Physiol.*, **1985**, *118*, 227-235.

[20]Ashihara, H. and Nobusawa, E., *Z. Pflanzenphysiol.*, **1981**, *104*, 443-458.

[21]Ashihara, H., Mitsui, K. and Ukaji, T., *Z. Naturforsch.*, **1987**, *42c*, 297-290.

[22]Amino, S., Fujimura, T. and Komamine, A., *Physiol. Plant.*, **1983**, *59*, 393-396.

[23]Ashihara, H., Li, X. N. and Ukaji, T., *Ann. Bot.*, **1988**, *61*, 225-232.

[24]Stasolla, C., Katahira, R., Thorpe, T. A. and Ashihara, H., *J. Plant Physiol.*, **2003**, *160*, 1271-1295.

[25]Mitsui, K. and Ashihara, H., *Plant Cell Physiol.*, **1988**, *29*, 1171-1183.

[26]Fujimura, T., *Plant Biotech. Rep.*, **2014,** *8*, 23-28.

[27]Stasolla, C., *Method. Mol. Biol.*, **2006**, *318*, 87-99.

Received: 13.10.2014.
Accepted: 05.11.2014.