A REVISION OF THE MEDIUM FOR SOMATIC EMBRYOGENESIS IN CARROT SUSPENSION CULTURE

Kiyoshi MASUDA, Yoshio KIKUTA and Yozo OKAZAWA

(Department of Botany, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan)

Received April 27, 1981

Introduction

Since Steward's first observation of occurrence of small plantlets with cotyledons and integrated root-shoot axis derived from unorganized cells in carrot cultures[7], somatic embryogenesis has been a favoured subject in tissue cultures and many works have been established from a morphological standpoint.

In principle somatic embryogenesis in part cell cultures offers a most ideal system for physiological studies on early developmental stage of morphogenesis. Among a wide range of plant cell cultures, it has been demonstrated that carrot cells in suspension cultures expressed their high embryogenic competence. However, for the investigations on physiological aspect of somatic embryogenesis, synchronized differentiation of the embryos with high frequency was required. HALPERIN[3] has succeeded partially in synchronized embryogenesis using suspension cultures of wild carrot, by means of subculturing relatively uniform cell clumps obtained by sieving to fresh medium in which the concentration of 2, 4-D was declined. This methods were further advanced by FUJIMURA and KOMAMINE[1,2] who investigated on the hormonal factors required for somatic embryogenesis. Nutritional factors being essential for the development of the carrot cultures have been examined in detail with respect to carbohydrates[5] and nitrogen sources[6].

This paper is described on the experiments designed to evaluate the influences of growth regulators, sugars and nitrogen sources on somatic embryogenesis in suspension cultures of domestic carrot and also pursued as to the revision of the medium being suitable for the rapid establishment of embryos with high frequency.

Materials and Methods

Plant Materials: Seeds of carrot (*Daucus carota* L., c v. Red Core Chantenay) were surface-sterilized with 0.4% aqueous Na-hypochlorite solution, rinsed with steril water and allowed to germinate on 0.6% agar (Difco, Bacto Agar) containing 2% sucrose for a week at 27°C under constant illumination (Toshiba, Type FLA 40 S/M, ca. 1500 lux). These seedlings were served as source of tissue explants. The hypocotyl segments of 5 mm long from the seedlings were grown on MS medium[4] which was modified by the concentrations of NH_4NO_3 and KNO_3 to 10.3 mM and 24.7 mM, respectively. Other organic addenda were thiamine-HCl (3 mg/l), nicotinic acid (5 mg/l), pyridoxine-HCl (0.5 mg/l), sucrose (20 g/l) and casein hydrolysate (500 mg/l, Difco. certified grade), and the medium was referred to as basal medium. As growth regulator, 2, 4-D at 0.9×10^{-6} M was supplemented to the medium for initiation and maintenance of the culture. After a month of cultivation, the calli were transferred to 80 ml of the liquid medium consisted of the same composition without agar, and cultured on a reciprocal shaker agitated 120 rpm, 1.0 inch stroke. The cultures were subcultured at intervals of 13 to 15 days.

Test Culture: The cells subcultured for 3 to 5 passages were subjected for the test culture. At the end of culture, cell suspensions were sieved with a 177 μm metal screen and then collected on a 44 μm one in order to eliminate the large cell clumps and vacuolated free cells. The cell clumps retained on a 44 μm screen were washed with the fresh medium twice by centrifugation at 80 g for 3 min and subsequently inoculated to glass tubes (15×130 mm) containing 2.0 ml of culture medium at a density of 1.3 μl packed cell volume per ml. These cultures were incubated in 10 rpm rotatory cultivator. The yields of embryos were determined by counting of plantlets and torpedo embryos. The dry weights were determined by following method: the cultured cells were collected and washed on Whatman GF/C glass filters, dried at 80°C overnight and weighed. These values were obtained at 14-day after inoculation.

Growth Regulators: As growth regulator, 2, 4-D (2, 4-Dichlorophenoxyacetic acid, Sigma), IAA (Indole-3-acetic acid, Merck), zeatin (Sigma), and GA (gibberellic acid, Kyowa Hakko) were separately supplemented to the basal medium at various concentrations. IAA and zeatin were filter-sterilized to avoid heat decomposition.

Nitrogen Sources: KNO_3 and NH_4Cl were used as inorganic nitrogen sources at various concentrations, but in return casein hydrolysate was

omitted from the basal medium and the level of potassium was adjusted by the addition of KCl up to 40 mM to prevent potassium shortage. Casein hydrolysate as organic nitrogen source was reinforced to the medium containing of inorganic nitrogen source.

Sugars: Various types of sugars at 58 mM or 116 mM were substituted for sucrose in the basal medium. The optimum concentration of sucrose for embryogenesis *in vitro* was also determined.

Time Course Experiment: Five milliliters of cell suspension were inoculated into 75 ml of the basal medium and the cultures were maintained on a reciprocal shaker. The number of embryos in aliquots of culture was determined under a microscope.

The media were adjusted to pH 5.8 with NaOH before autoclaving unless otherwise described. These cultures were maintained at 27°C under continuous fluorescent illumination. All the chemicals were purchased commercially from Wako Chemicals unless stated above.

Results and Discussion

Growth Regulators: Figure 1 shows that 2, 4-D and IAA promote the somatic embryogenesis of carrot suspension cultures about 25% at 10^{-10} M

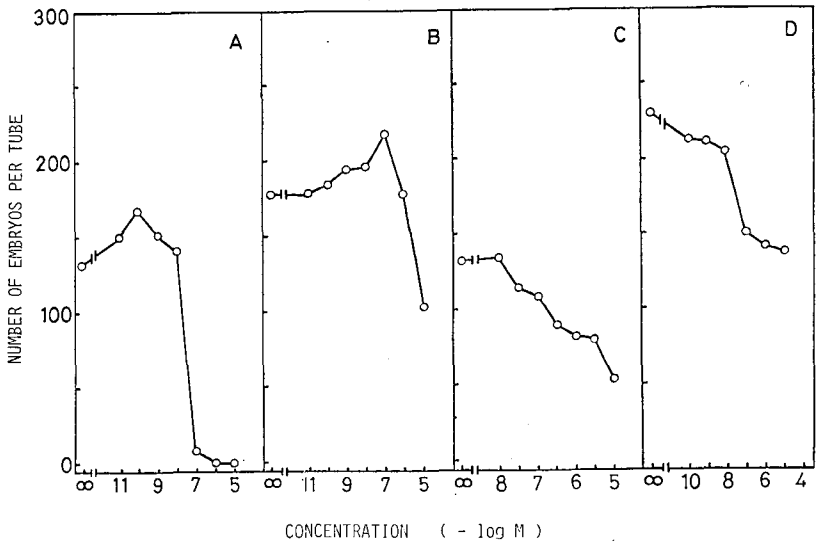

Fig. 1. Effects of 2, 4-D (A), IAA (B), zeatin (C) or GA (D) on the formation of embryos in carrot cell suspensions. Embryos in torpedo stage and plantlets were counted on the 14th day of culture.

and 10^{-7} M, respectively. Applications of higher concentrations of these auxins, however, decreased in the number of embryos. Embryogenesis was completely inhibited with the presence of 2, 4-D at 10^{-7} M. Gibberellic acid and zeatin showed the inhibition at the range from 10^{-8} M to 10^{-5} M. These results suggest that concentration of 2, 4-D lowered below 10^{-8} M is required for the embryogenesis in suspension cultures of carrot and that exogeneous supply of gibberellic acid and zeatin is not a essential factor.

Nitrogen Sources: The relative effectiveness of ammonium and nitrate as nitrogen sources supporting embryogenesis is summerized in Figure 2. The increase of KNO_3 concentration support the yield of embryos for any NH_4Cl level tested, however, KNO_3 as a sole source of nitrogen shows very low frequency of embryogenesis. Provided the concentration of KNO_3 was declined to 10 mM, NH_4Cl obviously inhibited somatic embryogenesis at 10 mM. These results reveal that the optimum concentration of KNO_3 lies around 40 mM and that the optimum concentration of NH_4Cl corresponds to 25%~30% of KNO_3 levels, but which display a tendency to vary accompanying with the changes in level of KNO_3.

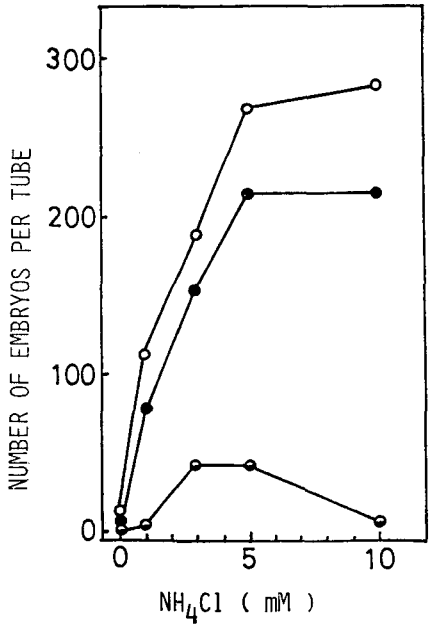

Fig. 2. Changes in number of embryos as a function of NH_4Cl level. Medium contained KNO_3 at 40 mM (○——○), 20 mM (●——●), or 10 mM (◒——◒). Determination was carried out as the same manner as Fig. 1.

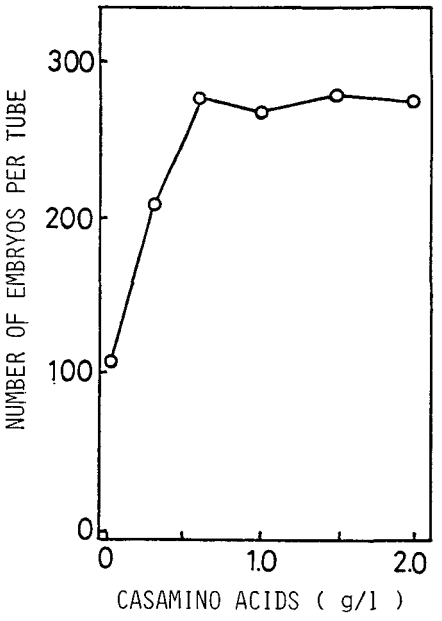

Fig. 3. Embryogenesis on medium containing different cocentrations of casein hydrolysate. Medium included both 10.3 mM NH_4NO_3 and 24.7 mM of KNO_3 as inorganic nitrogen sources.

Figure 3 shows an increase in the number of embryos due to the supplement of casein hydrolysate, where the medium includes 10 mM NH_4NO_3 and 24.7 mM KNO_3 as inorganic nitrogen sources. WETHERELL and DOUGALL[6] reported that casein hydrolysate elicited supporting effect on growth and embryogenesis as a sole nitrogen source while the compound was not as effective as ammonium when supplemented nitrate. However, the data presented here indicates that 600 mg/l of casein hydrolysate in the presence of ammonium at 10 mM increases the number of embryos over 150%. An evidence from the commercial information (Difco) that casein hydrolysate contains about 10% of nitrogen led us to believe that the medium with 600 mg of this per liter appears to correspond to about 4.3 mM nitrogen. To compare the availability of reduced nitrogen sources for the embryogenesis between inorganic and organic forms, cultures were performed on the different media at the equal level of nitrogen, *i. e.* 35 mM KNO_3 in combination with 10 mM NH_4Cl plus 600 mg/l of casein hydrolysate, and with 15 mM NH_4Cl alone. As far as can be considered it from the result that unlike in combination of casein hydrolysate a single application of NH_4Cl was liable to

retard the embryogenesis if exceeding 10 mM, it seems reasonable to assume that inorganic ammonium nitrogen is not applicable to stimulate as effective as organic nitrogen of casein hydrolysate, even at the equal level of reduced nitrogen. However, we failed to completely separate the processes between the growth and embryogenesis in carrot suspension cultures, suggesting that the further detailed analysis of the developmental process whether or not the influence of casein hydrolysate is required on embryogenesis *in vitro*.

Sugars: Table 1 shows that the frequencies of embryogenesis on the medium enriched separatley with glucose, fructose, mannose, fructose+glucose, and raffinose are considerably less than those occurred on either sucrose or maltose. Although the cultured carrot cells were ascertained to utilize galactose as a sole carbon source for their growth and embryogenesis as reported by VERMA and DOUGALL,[5] the least frequency of embryogenesis was recorded.

Fructose was only sugar to inhibit embryogenesis even at higher concentration, but the compound incited the embryogenesis and growth near the same extent as glucose or mannose provided its level lowered down to

TABLE 1. Effects of sugars on growth and embryogenesis in carrot cell suspensions. Culture volume, 2 ml/tube. Cultures were terminated after 14 days of incubation

Sugars	mM	Number of Embryos	Dry Weight (mg)	
			−2, 4-D	+2, 4-D*
Glucose	58	164±31	9.8±0.8	—
	116	176±26		
Fructose	58	168±14	9.0±0.2	8.9±0.1
	116	77± 1		
Galactose	58	65±11	3.1±0.5	2.4±0.5
	116	85± 3		
Mannose	58	164± 4	9.4±1.3	8.2±0.3
	116	185±35		
Sucrose	58	259±45	16.5±0.7	7.0±0.5
Maltose	58	225±29	16.3±1.1	5.2±0.6
Lactose	58	0	1.3±0.1	1.8±0.2
Fructose & Glucose	58	169±42	—	—
Raffinose	58	169±26	9.6±3.0	7.6±0.4

* Concentration of 2, 4-D: 0.9×10^{-6} M.

TABLE 2. Effect of sucrose on growth and embryogenesis in carrot cell suspension. Culture volume, 2 ml/tube. Experiments were terminated after 14 days of incubation

Sucrose (mM)	Number of Embryos per Tube	Dry Weight (mg/Tube)
0	0	0.7
15	83	4.0
29	189	8.9
58	304	15.8
117	161	13.7
234	38	10.1

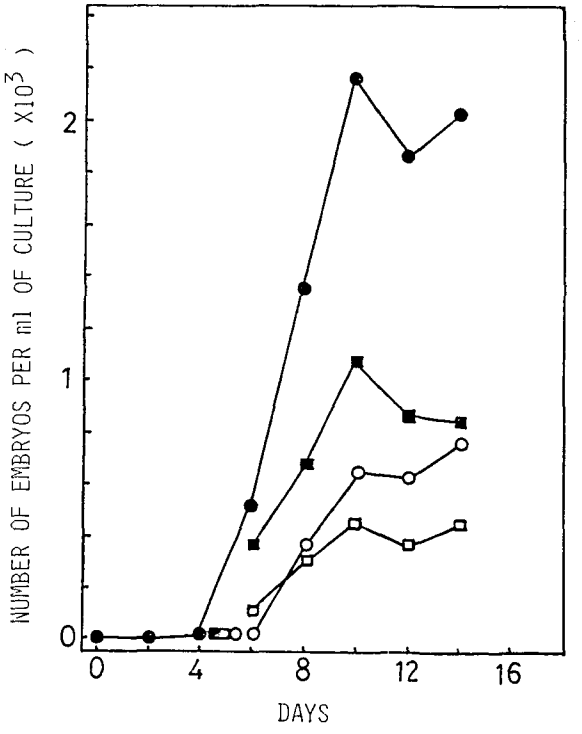

Fig. 4. Time course experiment of embryogenesis on the basal medium. Cultures were agitated by a reciprocal shaker. Aliquots of culture were sampled and the number of embryos were determined. Total embryos (●——●), globular embryos (■——■), heart shaped embryos (□——□), and torpedo embryos and plantlets (○——○).

58 mM. This result suggests that the inhibitory effects of autoclaved fructose may be little, if any, in carrot suspension cultures.

After 14 days of incubation, on the medium enriched with either sucrose or maltose, the lack of 2, 4-D enabled the growth of the culture to improve significantly as compared with the presence of 2, 4-D. The similar results were not observed in carrot cells grown on fructose, galactose,

TABLE 3. Revision of medium for carrot cell suspension culture

Mineral salts	
Macro elements	
Salts	mM
NH_4NO_3	10.3
KNO_3	24.7
$CaCl_2$	3.0
$MgSO_4$	1.5
KH_2PO_4	1.25
Na_2-EDTA	Na, 0.2[1)]
$FeSO_4$	Fe, 0.1[1)]
Micro elements	
Salts	μM
H_3BO_3	100
$MnSO_4$	100
$ZnSO_4$	30
KI	5.0
Na_2Mo_4	1.0
$CuSO_4$	0.1
$CoCl_2$	0.1

1) As Fe-EDTA

Organic components	mg/l
Sucrose	20,000
Casein hydrolysate	500
myo-Inositol	100
Thiamine-HCl	3
Nicotinic acid	5
Pyridoxin-HCl	0.5
2, 4-Dichlorophenoxyacetic acid (optional)	0.2

pH was adjusted to 5.8 with NaOH.

or mannose. The optimum concentration of sucrose for the growth and embryogenesis of the carrot cell suspension cultures is presented in Table 2 which shows the highest values of both embryos and dry weight at 58 mM.

Time Course: Time course of embryogenesis in the carrot cell suspension culture is shown in Figure 4. Inoculum consisted of cell clumps and vacuolated free cells (Fig. 5 a). Morphologically distinguishable embryos did not occur within 4 days after inoculation. Numerous embryos emerged rapidly thereafter. Many embryos arose from the peripheral layers of cell clumps which in turn acquired typical globular embryo (Fig. 5 b). Torpedo embryos were observed after 8 days of incubation (Fig. 5 c). About 40% of these embryos developed upto torpedo stage and plantlets until the end of culture (Fig. 5 d). However, no visible embryos were observed on the medium supplemented with 2, 4-D at 0.9×10^{-6} M.

The composition of the medium being suitable for rapid growth and embryogenesis in carrot cell suspension cultures is presented in Table 3.

Summary

Effects of growth regulators, sugars and nitrogen sources on growth and embryogenesis in carrot cell suspension cultures were examined. Although 2, 4-D and IAA showed slight promotion of embryogenesis at 10^{-10} M and 10^{-7} M, respectively, the application of the higher concentrations of these auxins revealed the decrease in number of embryos. Application of zeatin at the range from 10^{-8} M to 10^{-5} M and gibberellic acid from 10^{-10} M to 10^{-5} M showed no stimulating effect on the formation of embryos.

The optimum concentration of KNO_3 lay around 40 mM, while that of NH_4Cl varied with changing in the level of KNO_3. The application of casein hydrolysate increases in the number of embryos up to 160%.

Carrot cells could utilize galactose for their growth and embryogenesis. The most frequent embryogenesis and highest growth of them were recorded on the medium enriched with surcose and maltose without 2, 4-D. The optimum concentration of sucrose was 58 mM for growth and embryogenesis in carrot cell cultures.

From the observation of the time course experiment of embryogenesis, embryos were begun to emerge adventitiously about 4 days after inoculation of cell clumps to fresh medium, and the number of embryos reached to the maximum level after 8 days of incubation. Finally 40% of embryos succeeded to acquire torpedo embryos or plantlets.

Acknowledgement

This work was supported by Japan Society for the Promotion of Science to K. MASUDA and in part by Scientific Research Grant in Aid from the Ministry of Education of Japan.

Literature Cited

1. FUJIMURA, T. and KOMAMINE, A.: Effects of various growth regulators on the embryogenesis in a carrot cell suspension culture. *Plant Scs. Lett.* **5**: 359–364. 1975
2. FUJIMURA, T. and KOMAMINE, A: Synchronization of somatic embryogenesis in a carrot cell suspension culture. *Plant Physiol.* **64**: 162–164. 1979
3. HALPERIN, W.: Morphogenetic studies with partially synchronized cultures of carrot embryos. *Science* **146**: 408–409. 1964
4. MURASHIGE, T. and SKOOG, F.: Revised media for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15**: 473–497. 1962
5. VERMA, D. C. and DOUGALL, D. K.: Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. *Plant Physiol.* **59**: 81–85. 1977
6. WETHERELL, D. F. and DOUGALL, D. K.: Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiol. Plant.* **37**: 97–103. 1976
7. STEWARD, F.: Interpretations of the growth from free cells to carrot plants. *Am. J. Bot.* **45**: 709–713. 1958

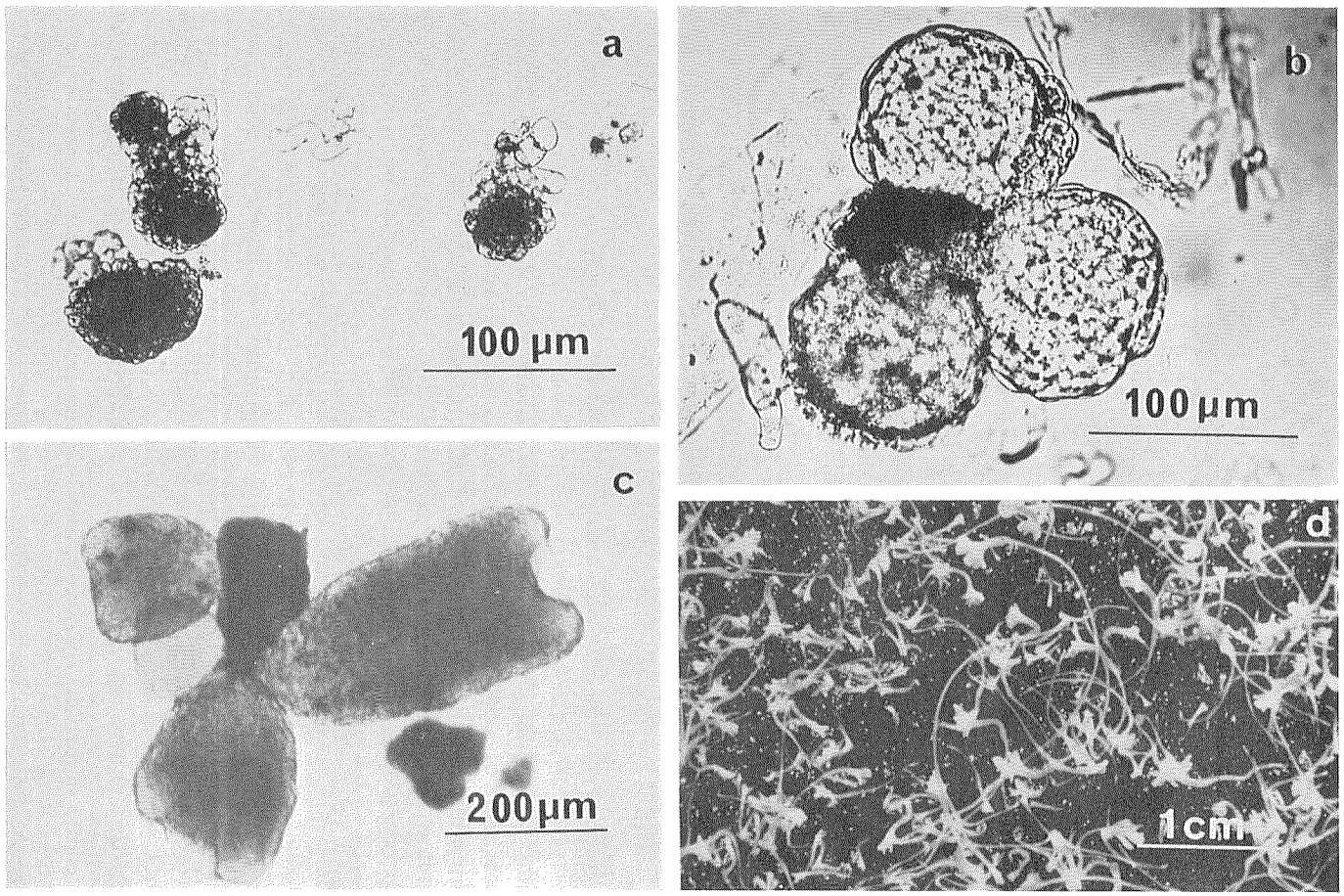

Fig. 5. Somatic embryogenesis and their development in carrot cell suspensions. a. Inoculum. b. Embroys in globular stage. c. Typical appearance of embryos at heart and torpedo stages. d. Numerous plantlets developed in 14 days of incubation.