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당근 모상근의 최적 영양 요구성

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Optimal Nutritional Requirements of Carrot Hairy Roots

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ABSTRACT

The physiological characteristics of carrot hairy roots and suspension cells were examined based on their nutritional requirements. Inorganic nutrient (phosphorous and ammonium) requirements of carrot hairy roots were similar to those of suspension cells. Optimal sucrose concentration for the growth of hairy roots (7%) was different from that of suspension cells (3%). Since suspension cells were more easily affected by the environmental condition, e.g., osmotic stress, than hairy roots which made the suitable growth condition for cells, it can be understood that optimal sucrose concentration for the growth of hairy roots was higher than that for the growth of the suspension cells. To investigate the roles of sucrose on the growth of hairy roots, the effects of sucrose on the fresh weight and dry weight was analysed by the addition of mannitol as an osmoticum. Sucrose acts also as an energy source for hairy roots rather than as an osmotic regulator, since the increase of dry weight was higher than that of fresh weight at the given sucrose concentration.

INTRODUCTION

In plant cell/tissue culture system, a variety of culture media have been reported to support the growth of plant cells. The component of plant cell culture medium can be classified as follows; 1) macro (nitrogen, phosphorous, sulfur, calcium and potassium) and micro nutrients, 2) organic nutrients (carbon and vitamin), 3) growth regulators (auxin, cytokinin and gibberellin) and 4) natural complexes (yeast extract, malt extract and peptone). Nitrate and ammonium are generally used as nitrogen sources in plant culture media. Generally, ammonium alone showed poor growth compared with nitrate alone (1). And the effects of media with a wide range of ammonium to nitrate ratios on the growth and metabolite production have been reported (2, 3). Nakagawa et al. (4), reported that the highest yield of berberine in *Thalictrum minus* was obtained when the ratio of nitrate to ammonium was 1:2, while maximal growth was observed at a ratio of 5:1. Also, the phosphate concentration in the medium can be an important parameter when controlling

the growth of plant cells and production of secondary metabolites. Obata-Sasamoto and Komanine (5) showed that media in which growth was restrained by low phosphate were favorable for the production of L-DOPA in callus cultures of *Stizolobium hassjoo*. Sucrose and its component monosaccharide, glucose and fructose, are usually used as the carbon sources. The sugar concentration giving maximum growth differs among species, but the growth rate and final biomass are affected by the initial sucrose concentration in the medium. Especially, sucrose used to be a limiting nutrient in the plant cell culture system. In general, growth regulators, auxin and/or cytokinin are added to the culture media. Addition of auxin is essential to induce and maintain the growth of plant cells, but the requirement for exogenous supply of cytokinin depends on the plant species. Skoog and Miller (6) reported the growth and morphogenesis in vitro, as controlled by ratio of auxin to cytokinin with *Nicotiana tabacum*. In secondary metabolite production, various effects of auxins and cytokinins have been reported. While auxins (NAA and IAA) stimulate anthocyanin formation in suspension cultures of *C. populus* (7), cytokinin (kinetin) inhibits the carotenoid synthesis in carrot cultures (8).

There are many reports on the effect of medium nutrient in the plant cell suspension culture, but few comparative studies on the nutrient effect in the hairy root system have been conducted so far. It is important to know the differences of nutritional requirements between hairy roots and suspension cells for cultivation of hairy roots. Therefore, in this study, the physiological characteristics of hairy roots were investigated and the differences between hairy roots and suspension cells were examined based on their nutritional requirements.

MATERIALS AND METHOD

Materials

Carrot hairy roots and suspension cells (*Daucus carota*) were obtained from Chunnam National University and Department of Biology in Seoul National University, respectively.

Hormones, indoleacetic acid (IAA), naphthaleneacetic acid (NAA) and benzylamino purine (BAP) were purchased from Sigma Chemical Co. (U. S. A.).

Culture Condition

Carrot hairy roots were maintained in Murashige & Skoog's medium containing 30 g/ℓ (0.1461 mM) sucrose. The hairy roots were cultivated in 250 ml Erlenmeyer flask with 50 ml medium on a rotary shaker at 120 rpm under the dark condition. The temperature was 27℃, and the medium pH was adjusted to 5.8 with 2N NaOH followed by autoclaving at 121℃ for 15 minutes.

Analysis

Root Mass Measurement

Fresh hairy root weight was measured after washing with distilled water and vacuum filtration through Whatman No. 2 filter paper. Dry weight was measured after drying the roots at 80℃ for 2 days. Two or three replicates of flasks were taken for analysis.

Sucrose

Sucrose concentration was measured colorimetrically by DNS method (9) using spectrophotometer (UVIKON 930, Kontron, U. S. A.).

Microscopic Observation

For microscopic observation, hairy roots were taken at the 11th day of cultivation. The roots were cut 1 cm from the end, where active cell division was occurred. The samples were fixed in 70% ethanol solution and placed on clean glass slides. After staining with 0.1% methylene blue, the samples were squeezed for a monolayer layout. The observation was conducted using a photo microscope (OPTIPHOT-II and UFX-DX, Nikon, Japan).

RESULTS AND DISCUSSION

Effect of Nutrient Concentration on the Growth of Carrot Hairy Roots

The effect of phosphorous on the hairy root growth was investigated. Phosphorous is an important nutritional element for plant cells since it is required for nucleic acid metabolism and energy metabolism as a component of UDP, ADP, ATP etc. However, as shown in Table 1, the highest root mass was obtained at the control case and significant difference of the growth by the phosphate concentration was not detected. Similar result was obtained in the carrot plant cell culture system (10).

Table 1. Effect of initial phosphate concentration on the growth of carrot hairy roots. Samples were taken at the 11th day of cultivation.

KH_2PO_4 conc. (mg/ℓ)	Fresh weight (g/ℓ)	Dry weight (g/ℓ)
100	311.7	14.58
150	289.9	14.07
170*	352.2	15.08
200	348.7	14.71
250	332.8	14.62
300	318.4	14.83

* control

Table 2. Effect of ammonium and nitrate concentration on the growth of carrot hairy roots. Samples were taken at the 11th day of cultivation.

Ratio of ammonium to nitrate	Total nitrogen conc. (mM)	Fresh weight (g/ℓ)	Dry weight (g/ℓ)
1:0	30	151.4	8.25
0:1	30	378.1	18.02
1:0	60	93.7	3.66
2:1	60	317.2	14.41
1:1	60	360.5	16.78
1:2*	60	360.5	15.92
0:1	60	393.6	17.32
1:0	90	86.5	4.62
1:1	90	259.5	14.65
1:2	90	216.3	14.33
0:1	90	324.4	16.88

* control

In plant cell/tissue culture system, ammonium and nitrate are used as nitrogen sources. The effect of nitrogen concentration and ratio of the two components on the growth of hairy roots were investigated. As shown in Table 2, optimal total nitrogen concentration for growth of hairy roots was 60 mM, and the growth of hairy roots was severely inhibited when the ammonium was supplied as a sole nitrogen source. Generally, ammonium ion as the sole nitrogen source is unsuitable, probably because the pH of the medium drops during the cultivation under such situation (11). This drop in pH which inhibits the availability of nitrogen has been reported for the rice (12) and carrot (13) suspension cell cultures. Although, ammonium is not adequate as the sole nitrogen source, it has been reported a small amount of ammonium may be essential for the better growth (14). In previous report of carrot suspension cell cultures (10), optimal total nitrogen concentration was 60 mM, and the ratio of ammonium to nitrate for cell growth was 1 : 1. But, in the carrot hairy root culture system, it seems that the root growth was not affected by the ratio of the two nitrogen components, although the highest root mass was obtained in the MS medium containing nitrate as a sole nitrogen source. The effect of natural complexes and the growth regulator on the growth of hairy roots was examined. When various natural complexes which are known to be the growth stimulator were tested, significant effect was not detected on the growth of hairy roots as shown in Table 3. Indole acetic acid (IAA) and naphthaleneacetic acid (NAA) were tested as an auxin, and the benzylamino purine (BAP) was tested as a cytokinin. As shown in Table 4, in all cases, the growth regulators did not stimulate the hairy root growth. Since hairy roots synthesize endogenous auxin, it seems that the exogenous supply was not necessary.

Table 3. Effect of natural complexes on the growth of carrot hairy roots. Samples were taken at the 11th day of cultivation.

Natural complex	Fresh weight (g/ℓ)	Dry weight (g/ℓ)
control	303.2	15.26
peptone	278.6	17.37
yeast extract	247.6	16.60
malt extract	278.6	15.96

Table 4. Effect of hormone on the growth of carrot hairy roots. Samples were taken at the 12th day of cultivation.

Hormone	Fresh weight (g/ℓ)	Dry weight (g/ℓ)
control	352.2	15.08
NAA	431.6	15.07
IAA	369.1	15.26
BAP	370.2	14.93

Effect of Initial Sucrose Concentration on the Growth of Hairy Roots

The effect of initial sucrose concentration on the growth of carrot hairy roots was investigated. Fig. 1(a) shows the time courses of fresh weight of hairy roots. Maximum growth of hairy roots was observed in the MS medium containing 7% sucrose and the highest root mass was obtained after cultivating 12 days. The growth rate was increased with the increasing sucrose concentration, but the growth rate was declined over 7% sucrose medium. Fig. 1(b) shows the batch profiles of dry weight of hairy roots. The 7% sucrose concentration gave also the maximal growth of hairy root dry weight as for the fresh weight. The results differ from that obtained in the carrot suspension cell cultures (15). The optimal sucrose concentration for carrot suspension cultures was 3%. The growth of suspension cells was inhibited as the sucrose concentration was increased. Generally, the role of sucrose in plant cell culture system is not only an energy source for growth and production but also an osmotic regulator of the medium (16, 17). The specific growth rate is

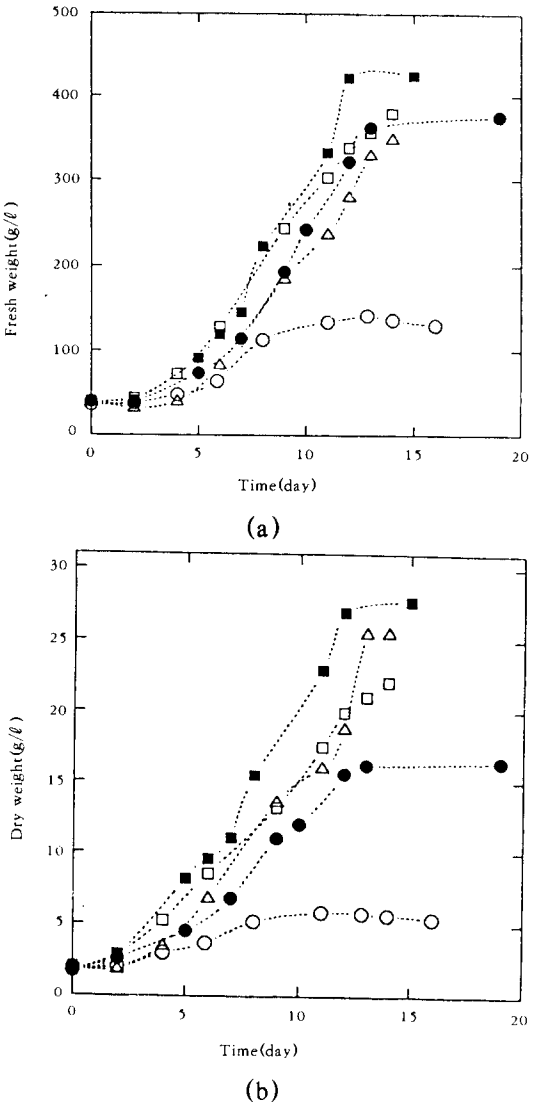


Fig. 1. Time courses of (a) fresh weight, (b) dry weight in MS medium for initial sucrose concentrations.
○:1% sucrose, ●:3% sucrose, □:5% sucrose, ■:7% sucrose, △:9% sucrose

a function of sucrose concentration. Higher sucrose concentration usually inhibits cell growth in suspension culture. High initial level of sucrose raised the medium osmotic pressure and thus caused physiological change of the cells. Suspen-

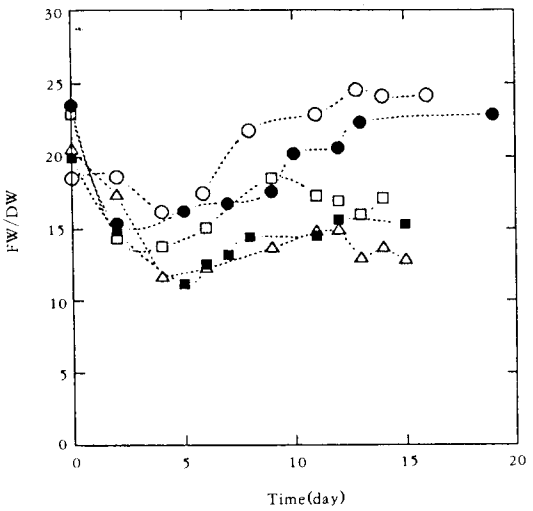


Fig. 2. Time courses of FW/DW in MS medium for various initial sucrose concentrations. ○:1% sucrose, ●:3% sucrose, □:5% sucrose, ■:7% sucrose, △:9% sucrose

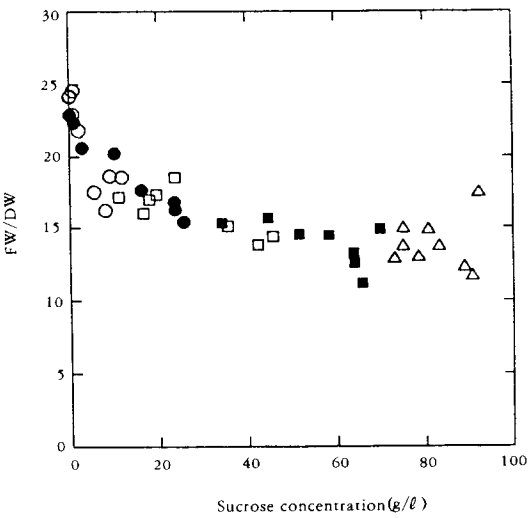


Fig. 3. Relationship between FW/DW and sucrose concentration in MS medium for various initial sucrose concentrations. ○:1% sucrose, ●:3% sucrose, □:5% sucrose, ■:7% sucrose, △:9% sucrose

sion cells can be easily affected by the environmental conditions (e.g., osmotic pressure, nutrient concentrations etc.) once exposed. For *C. blumei*, the plasmolysis was observed at 75 g/ℓ sucrose (18). However, hairy roots, surrounded by the epidermal cells as an organ, are able to make the extracellular environment by the cell-to-cell interaction. Since the extracellular environment plays a buffering action, suitable growth condition is maintained in cellular level. Optimal sucrose concentration for growth of hairy roots was therefore higher than that for suspension cells.

FW/DW (fresh weight/dry weight) can be used as a cell size index or index of water content. Kimball et al. (17) showed that cell size decreased with the increasing of sucrose, glucose, mannitol, or sorbitol concentration in soybean culture. And in the carrot cell suspension culture, similar results were reported for various sucrose concentrations (15). As shown in Fig. 2, FW/DW increased during the cultivation, and the increased steeply at the low sucrose concentration region as shown in Fig. 3. The specific growth rates of hairy roots for various sucrose concentration medium are shown in Table 5. In the case of 9% sucrose medium, the growth rate of dry weight was higher compared to that in the case of 5% sucrose medium, although the growth rate of fresh weight was lower than that in the 5% sucrose medium. The specific growth rate of hairy roots based on dry weight in 9% sucrose medium was decreased about 4% compared to that in 7% sucrose medium, while the specific growth rate based on fresh weight was decreased about 10%.

Table 5. Effect of initial sucrose concentration on the growth of carrot hairy roots.

Sucrose concentration (g/ℓ)	Specific growth rate (FW:day^{-1})	Specific growth rate (DW:day^{-1})
10	0.124	0.101
30	0.170	0.163
50	0.167	0.171
70	0.173	0.181
90	0.156	0.173

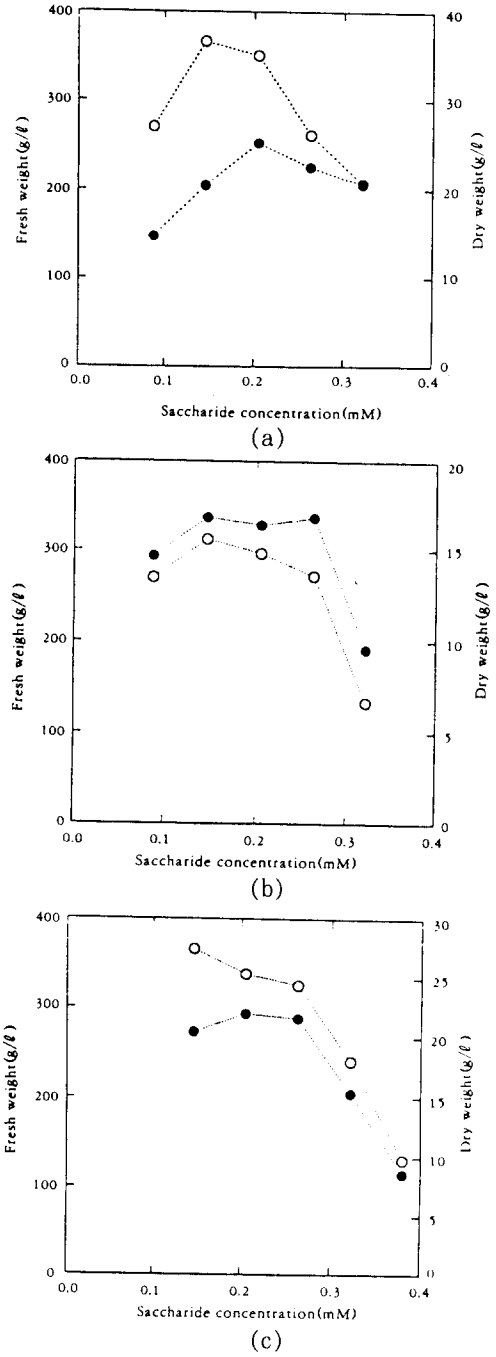


Fig. 4. Effect of initial saccharide concentration on the final hairy root weight.
(a) sucrose, (b) sucrose (0.146mM)+mannitol, (c) sucrose (0.2045mM)+mannitol
○:fresh weight, ●:dry weight

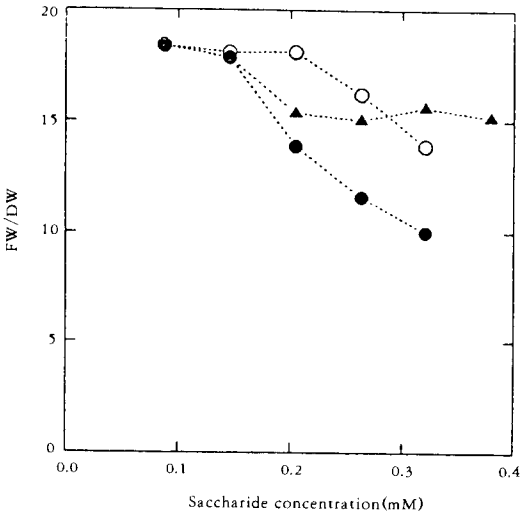


Fig. 5. Relationship between FW/DW and saccharide concentration in MS medium for various initial saccharide concentrations.
●:sucrose, ○:sucrose (0.1461mM)+mannitol, ▲:sucrose (0.2045mM)+mannitol

Role of Sucrose in Hairy Root Culture System

To investigate the role of sucrose in the hairy roots further, the effects of sucrose concentration on fresh weight and dry weight were examined. Since the increase of water content in the cells led to high fresh weight, osmotic regulator affected mainly the fresh weight. To examine sucrose as a potential osmotic regulator, the effect of sucrose concentration on hairy root weight was investigated at the given osmotic pressure region. In this experiment, the osmotic pressure which can be determined by the concentration of the medium was adjusted with the unmetabolized osmoticum, mannitol.

In Fig. 4(a), final hairy root weight shows strong dependence on the sucrose concentration in the medium. As shown in Fig. 4(b) and 4(c), since fresh weight and dry weight dropped at the high saccharide (sucrose+mannitol) concentration, it seems that mannitol played a role of osmotic regulator. But, in the case where the initial sucrose concentration was fixed, the increase of final root weight shown in the Fig. 4(a) was not

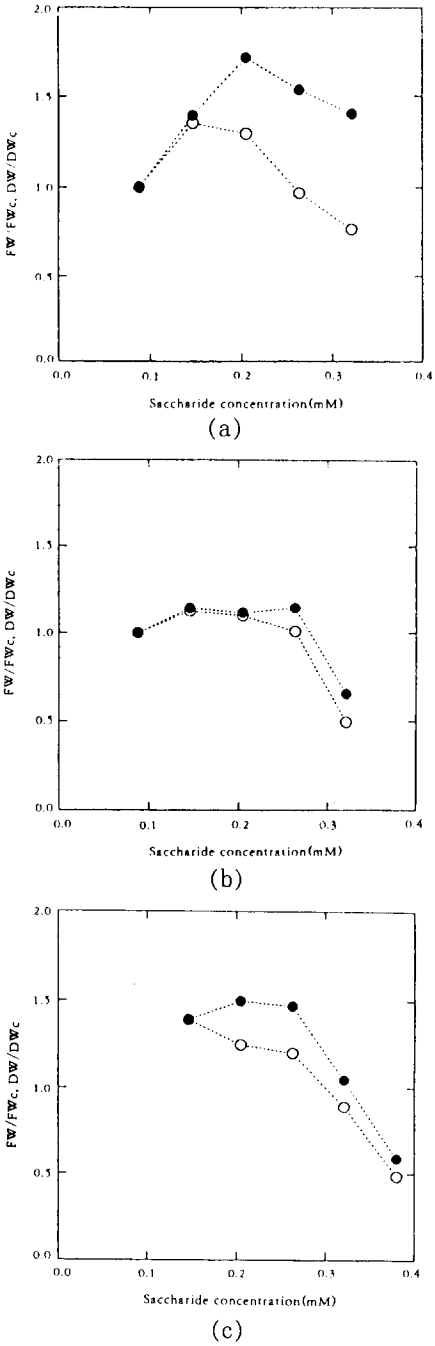
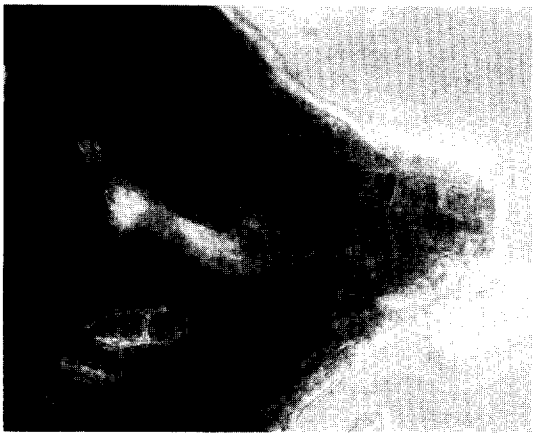


Fig. 6. Effect of initial saccharide concentration on the relative fresh weight and relative dry weight. (a) sucrose, (b) sucrose (0.1461mM)+mannitol, (c) sucrose (0.2045mM)+mannitol ○:fresh weight, ●:dry weight

observed. Higher final root weight was obtained at the high initial sucrose concentration compared to that of low initial sucrose concentration. It is therefore concluded that sucrose stimulated the growth of hairy roots in the limited range. The profiles of FW/DW are shown in Fig. 5 where the lowest values of FW/DW were observed using the sucrose alone. This phenomena is caused by the fact that the increase in dry weight was larger than the decrease in fresh weight. As shown in Fig. 6(a), since relative dry weight (final dry weight compared to that in control experiment) was higher than that of fresh weight at the given sucrose concentration, sucrose concentration affects the dry weight rather than the fresh weight. In the case where the initial sucrose concentration was fixed, there is little difference between relative dry weight and relative fresh weight as shown in Fig. 6(b) and 6(c). Hence, high sucrose concentration stimulated the dry weight growth, for sucrose played a role as an energy source rather than as an osmotic regulator in hairy root culture system. To confirm this result, microscopic observation was performed. Two hairy roots, one cultivated in low sucrose concentration (3%) and the other cultivated in high sucrose concentration (7%), were chosen for analysis.

As shown in Fig. 7, the cells in root tips, where active cell division occurs, showed small size and little differences between those in different sucrose concentration. Whereas the upper part of the roots where only cell enlargement occurs, the cells cultivated in high sucrose concentration were larger and the roots were as well thick as shown in Fig. 8. This result is the contrary result to the previous reports asserting the cells cultivated in lower sucrose concentration were larger than that in higher sucrose concentration on the basis that the water was uptaken into the cells under low osmotic pressure. But the fact that the cells cultivated in higher sucrose concentration were larger implies that hairy roots use sucrose as an energy source and build the cellular structure. In general, the increase of biomass is ac-

(a)



(b)

Fig. 7. The photograph of root tip cells of hairy roots for various sucrose concentrations (× 1,000). (a) 3% sucrose, (b) 7% sucrose

(a)

(b)

Fig. 8. The photograph of hairy roots for various sucrose concentrations (× 400). (a) 3% sucrose, (b) 7% sucrose

counted for by enlargement of the cells and increase of cell number, the higher sucrose concentration caused the cells grow larger which in turn caused the increase of dry weight.

요 약

당근 모상근의 생리학적 특성을 기존의 현탁배양세포와 영양 요구성의 관점에서 비교, 고찰하였다. 인산염과 질소원 등 무기 영양분의 영향은 당근현탁세포에서와 동일하였고 모상근 성장의 최적 sucrose 농도는 7%로, 3% sucrose 농도에서 최대 성장을 보인 현탁세포와는 다른 결과를 나타내었다. 이는 현탁배양세포는 미분화된 상태로 세포 각각이 외부 환경과 접촉하고 있으므로, 분화되어 기관 스스로 세포 성장에 적합한 환경을 만드는 모상근보다 외부 환경에 민감하게 작용하여 나타나는 현상으로 해석되었다. Sucrose가 모상근의 성장에 미치는 기작을 밝히기 위하여 삼투압 조절제인 mannitol을 배지에 첨가하여 sucrose 농도가 생체중량과 건조중량 각각에 미치는 영향을 고찰하였다. 모상근

의 성장은 배지 내 sucrose 농도에 영향을 받았고, sucrose 농도가 동일할 때 건조중량 증가는 생체중량 증가보다 크게 나타났다. 따라서 모상근 배양계에서 sucrose는 삼투압 조절제로서의 역할보다 주로 에너지원의 역할을 하는 것으로 판단되었다.

NOMENCLATURE

FW/DW : cell size index (fresh weight/dry weight)

FW/FWc : relative fresh weight
(fresh weight in various sucrose concentration/fresh weight in control experiment)

DW/DWc : relative dry weight
(dry weight in various sucrose concentration/dry weight in control experiment)

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