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Association between Water Status and Sucrose Metabolism in Cell Suspension Culture of Carrot

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Abstract

This work was undertaken to investigate the relationship between water status and sucrose metabolism of suspension culture of carrot (*Daucus carota* L.) cells. Embryogenic callus was induced on carrot hypocotyl *in vitro*. The cell suspension culture was then produced from this callus.

In suspension culture, water potential and pH of the culture solution were decreased immediately after culture initiation to −0.62 MPa and 4.5 respectively, but gradually increased from 2 days after culture initiation. Sucrose in suspension culture solution was found to be immediately hydrolyzed into glucose and fructose after the initiation of culture and disappeared within 2 days from culture initiation. The disappearance of sucrose from the culture solution within 2 days of culture initiation corresponds with increases in the activity of cell-wall-bound acid invertase. These results indicate that the decline of water potential of the culture solution at the early stages of carrot suspension culture was associated with sucrose metabolism as a result of an increase in acid invertase activity.

1. Introduction

The use of suspension culture in the production of embryogenic callus leading to somatic embryogenesis is a well established technique (George *et al.*, 1987; Taji *et al.*, 1997). It has been shown that the water potential of the medium decreased when embryogenic callus of carrot was cultured in auxin-free liquid medium (Nonami *et al.* 1995). However, the reason for this decline was not investigated by Nonami and co-workers (Nonami *et al.* 1995).

Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium, which is widely used in suspension cultures, contains several inorganic compounds, and small quantities of some organic compounds, as well as sucrose. We think that the decline in water potential is solely caused by sucrose breakdown, because the inorganic compounds are almost always solubilized in water in the culture solution. Changes in the amount of other organic compounds, such as vitamins and growth factors, may be negligible to water status. Wang and Staba (1963) working on tissue-cultured spearmint stem and Dormer and Street (1949) on tissue-cultured tomato root found sucrose degradation within several days after culture initiation. However, their work differs from the work reported here in that they neither investigated the influence of sucrose degradation on the water status of tissue-cultured solution, nor the trigger for the breakdown of sucrose. It has been shown that the sucrose separation is attributed to sucrose metabolism by acid invertase (EC 3.2.1.26) (Parr *et al.* 1976; Stommel and Simon, 1989). However, the correlation between acid invertase activity, sucrose metabolism and the water status of the cell suspension culture in carrot has not been established.

Since the growth of cells *in vitro* and some plant developmental processes are pH dependent (Smith and Krikorian, 1990), this correlation was also explored in relation to pH changes in the culture medium. Changes in media pH have occurred in a number of plants after subculturing (Williams *et al.* 1990; Shigeta *et al.*, 1996).

2. Materials and methods

2.1. Plant materials and culture conditions

The seeds of carrot (*Daucus carota* L. cv. Kuroda

-gosun; Takii and Company, Kyoto, Japan) were surface sterilized in a 1% (v v^{-1}) sodium hypochlorite solution for 10 min, followed by two rinses in sterile water. The seeds were sown on sterile gelrite-based (2 g l^{-1}) MS medium containing 30g l^{-1} sucrose and were incubated in the dark at 25 ℃. The carrot seeds germinated within one week, and 5-8 mm hypocotyl segments were transplanted to MS medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2 g l^{-1} gelrite and 30 g l^{-1} sucrose. The pH of the culture medium was set at 5.7 using 1N NaOH. Within a month, embryogenic callus had formed on the hypocotyl segments.

For suspension culture, the same culture medium as indicated above was used without the addition of gelrite. However in a concurrent experiment sucrose was replaced by 30g l^{-1} glucose and 30 g l^{-1} fructose. Each 100 m*l* flask contained 30 m*l* of the liquid medium. In order to avoid the effect of autoclaving on the integrity of culture medium (Wang and Hsiao, 1995), the medium was filter sterilized using first a 0.8 μm and then 0.22 μm filter (Millipore Corp., Bedford, MA, USA).

The embryogenic callus grown on the gelrite-based medium was transferred into the liquid medium one week prior to experimentation so that the callus could be acclimatized to the new liquid environment. The culture was incubated at 25 ℃ in the dark on an orbital shaker set at 100 rpm.

The callus from suspension culture was then filtered through a 500 μm metal mesh. The filtrate was transferred into fresh liquid medium prior to measurements. Cell density in each flask was 1 m*l* of packed cell volume. Samples of suspension culture were taken at 0, 1, 2, 3, 5, 10, 15, 20 and 25 days after initiation. The pH of the suspension culture was measured using a pH meter (HM-30S, TOA Electronics Ltd., Tokyo, Japan). Packed cell volume was determined by centrifuging the cells at 150xg.

2.2. Measurements of the liquid medium water status

Water potentials of the culture solution were determined using an isopiestic thermocouple psychrometer (Model-3, Isopiestic Psychrometry Ltd., DE, USA)(Boyer and Knipling, 1965). A thermocouple chamber was coated with melted and resolidified petrolatum (Boyer, 1967). Aliquots of the suspension culture solution were then dispensed into the sampling chamber.

2.3. Determination of sugar content

The sugar composition in the cell culture solution was analyzed by high performance liquid chromatograghy (HPLC). The culture solution was filtered through a 0.45 μm filter (Chromato disc 13A, Kurabo, Osaka, Japan). A 5 μl sample was injected into an HPLC (LC-10AD, Shimadzu Co., Kyoto, Japan) equipped with a refractive index detector (RID-6A, Shimadzu Co., Kyoto, Japan). Sugars were separated using a TSKgel Amide80 column (Tosoh, Tokyo, Japan) maintained at 80 ℃ with 80 % acetonitrile as the solvent at a flow rate of 1.0 m*l* min^{-1}. Glucose, fructose and sucrose were identified and quantified by comparison with peaks produced by a known standard sugar solution with a reporting integrator (C-R7A, Shimadzu Co., Kyoto, Japan).

2.4. Assays of enzymatic activity

For assays of acid invertase activity, plant samples were homogenized in a medium containing 50 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-KOH (pH 8.3), 2 mM Ethylenediaminetetraacetic acid (EDTA), 2 mM O,O'-Bis(2-aminoethyl) ethyleneglycol N,N,N',N'-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM $_{DL}$-Dithiothreitol (DTT) and centrifuged at 15,000xg for 5 min at 4 ℃.

The supernatant was dialyzed against a 0.02 M sodium acetate solution (pH4.5) using a cellulose tubing (Shiraimatsu, Tokyo, Japan) over night at 4 ℃, then used for vacuolar acid invertase analysis.

The pellet was re-washed with the homogenizing medium and centrifuged again. The cell-wall-bound acid invertase was solubilised from the washed pellet by shaking for 3 hours at 4 ℃ with a homogenizing medium containing 1 M NaCl. The mixture was centrifuged at 12,000xg for 5 min at 4 ℃. The resultant soluble fraction was used for assays without desalting.

The culture solution used for suspension was fractionated with $(NH_4)_2SO_4$. Ammonium sulfate was added until saturation. The whole was then centrifuged at 5,000xg for 5 min at 4 ℃. The resultant precipitate was solubilized in 5 m*l* homogenizing medium and used for assays without desalting.

For the assay of acid invertase activity, a solution containing 0.5 M sodium acetate (pH 4.5), 3 % (w v^{-1}) sucrose and the soluble fraction, was incubated at 37 ℃ for 15 min, heated at 85 ℃ for 2 min and then neutralized by addition of 1 M KOH. The assay solution treated as above, but without incubation, was used as a control. The concentration of glucose was then determined with an F-kit (Boehringer-Mannheim GmbH, Mannheim, Germany).

3. Results

3.1. Water status and pH of culture solution

When cells from embryogenic callus were transferred to the liquid medium containing 2,4-D, the water potential of the medium decreased immediately and reached -0.62 MPa by day 2 **(Fig. 1A)**. The water potential then began to increase and stabilized at approximately -0.13 MPa by 20 days after commencement of the experiments. The culture solution containing no cells did not alter in the 25 days duration of the experiment **(Fig. 1A)**.

The pH of the medium decreased to 4.5 by day 2 after culture initiation and then began to increase gradually throughout the experimental period **(Fig. 1B)**. The pH of the solution at 15 days after culture initiation was 5.6 which was almost the same as initial medium pH. The pH of culture solution containing no cells did not alter throughout the experimental period **(Fig. 1B)**.

The packed cell volume remained unchanged until approximately day 5, after which time it increased gradually **(Fig. 1C)**.

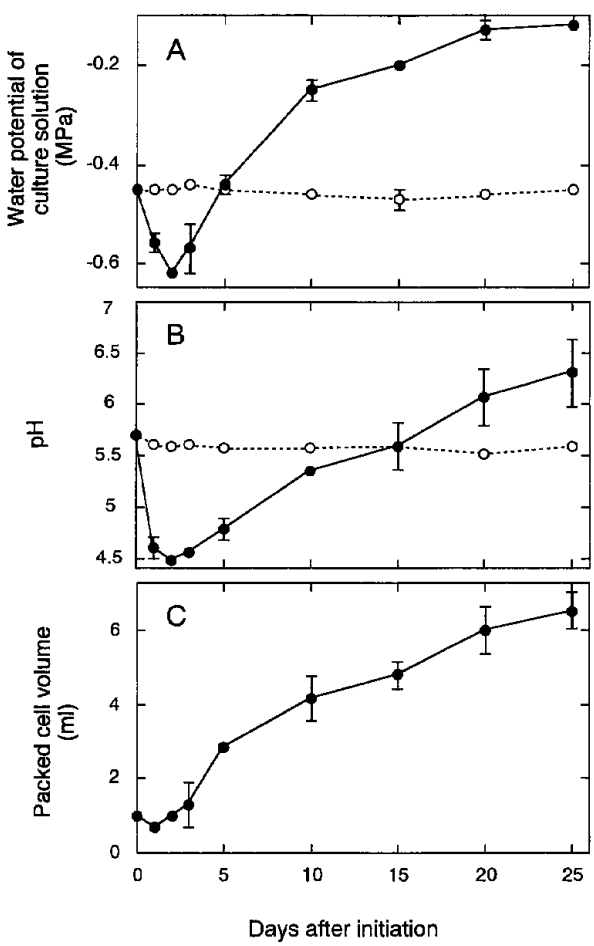

Fig. 1. Changes in water potential (A), pH (B) and packed cell volume (C) in cell suspension culture of carrot. In A and B, open circles (○) indicate culture solution without carrot cells and closed circles (●) culture solution containing carrot cells. Each point is the mean of at least 3 determinations. Vertical bars indicate standard deviation. Bars smaller than symbols were hidden within.

3.2. Sugar content in culture solution

Sucrose concentration reached almost zero within 2 days from culture initiation **(Fig. 2)**. This corresponded to an increase in the concentration of glucose (67 mM) and fructose (89 mM). Thereafter, the concentrations of glucose and fructose declined and reached almost zero by the 20th day after culture initiation **(Fig. 2)**.

3.3. Changes of invertase activity

The activity of cell-wall-bound invertase increased rapidly within 24 hours after culture initiation **(Fig. 3)** and peaked to 5.5 μmol · gFW^{-1} hour^{-1} by day 2. The acid invertase activity then declined to 2.9 μmol · gFW^{-1} hour^{-1} by day 3 and remained unchanged throughout the remainder of the experimental period **(Fig. 3)**.

When carrot cells cultured in MS solution containing 30 g l^{-1} glucose and 30 g l^{-1} fructose instead of sucrose, the cell-wall-bound acid invertase activity reached 3.0 μ mol gFW^{-1}hour^{-1} (5.5 μ mol gFW^{-1} hour^{-1} for sucrose treatment) at 2 days after initiation **(Table 1)**.

No acid invertase activitiy was detected in the soluble fraction of callus and culture solution in our experiments (data not shown).

Using an auxin-free medium instead of a 2,4-D rich medium similar results for water potential, pH,

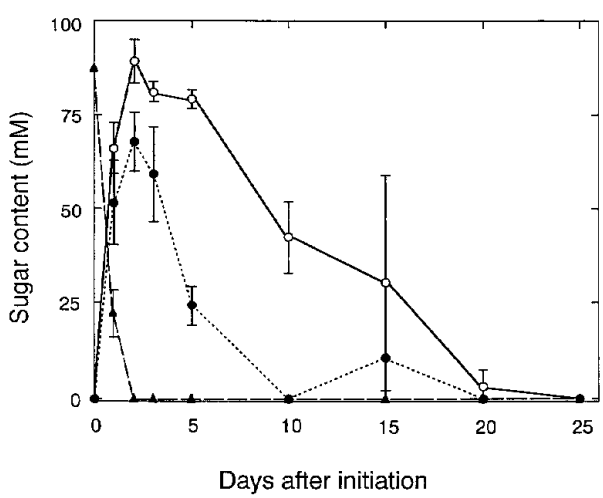

Fig. 2. Changes in sugar contents (● glucose, ○ fructose, and ▲ sucrose) in cell suspension culture of carrot. Each point is the mean of at least 3 determinations. Vertical bars indicate standard deviation. Bars smaller than symbols were hidden within.

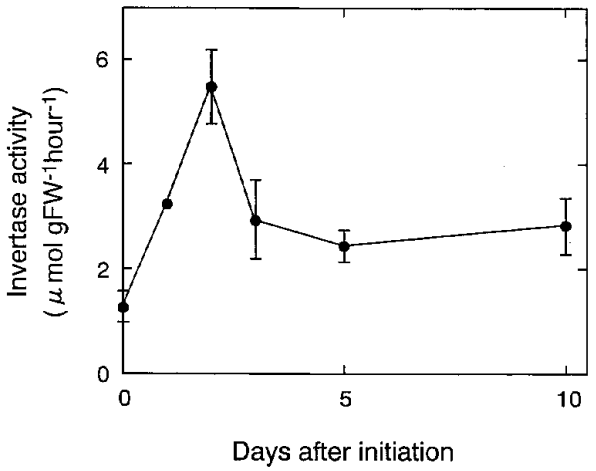

Fig. 3. Changes in cell wall-bound invertase activity in cell suspension culture of carrot. Each point is the mean of at least 3 determinations. Vertical bars indicate standard deviation. Bars smaller than symbols were hidden within.

packed cell volume, sugar changes and activity of acid invertase were obtained (data not shown).

4. Discussion

To our knowledge this is the first report showing the relationship between water status and sucrose metabolism in cell suspension cultures obtained from embryogenic callus in carrot. Substantial differences in the water potential of cells and the medium were obtained during the course of experiments reported here. The water potential of liquid medium almost equilibrates with that of the suspending callus (Nonami *et al.* 1995). Ikeda *et al.* (1999a, b) observed that the water potential of agar-based culture media were almost the same as those of soybean and carnation calli tissue grown on those media. Thus, in this experiment, the changes of water potential in liquid medium indicate changes of water potential in the suspended cells. The increase in packed cell volume observed after day 5 from culture initiation **(Fig. 1C)** may be due to cell division and cell expansion, which ultimately accompanies uptake and metabolize nutrient and water from the solution. This in turn increases the water potential of the liquid medium **(Fig. 1A)**.

The drop in pH to 4.5 at the initial stages of culture in our system is similar to that reported in *Ptilotus* shoot on agar-based medium (Williams *et al.* 1990). This pH has been shown to be the most favorable pH for acid invertase activity (Ricardo and ap Rees, 1970), and agrees with our results.

Water potential of the medium containing carrot cells reached -0.1 MPa by day 25, indicating that the culture solution contained a small amount of solutes by this stage. The increase in water potential must be a function of cell number and volume which increases solute uptake from the solution. Since a major factor in the pH change is the balance between NO_3^- and $NH4^+$ supply and uptake (Dougall, 1980), an increase in pH after 5 days of culture initiation may also be the result of differential NO_3^- and NH_4^+ uptake. The fluctuation in pH after day 15 was due to the low buffering capacity of a solution with low solute. The increase in water potential of medium supports this idea.

The cell-wall-bound acid invertase activity peaked by day 2 after initiation and then decreased **(Fig. 3)**. This coincides with the breakdown of sucrose to glucose and fructose, which also peaked by day 2 **(Fig. 2)**. The decline in acid invertase activity may be the result of a feed back regulation by sucrose level. Why acid invertase activity increases in culture medium by day 2 and subsequently declines is not clear. The sucrose in embryogenic callus medium was exhausted within 7 days when these calli, were separated into cells and when the resultant cells were transferred to fresh medium containing sucrose (see **Materials and methods**). We, thus, believe in such an environment the cell-wall-bound acid invertase activity was re-initiated and remained high until all sucrose was consumed. The initiation of cell-wall-bound invertase activity by sucrose was also reported in *Chenopodium rubrum* (Roitsch *et al.*, 1995) and sugar beet (Masuda *et al.*, 1988) cell suspension culture. Furthermore, when carrot cells cultured in MS solution containing 30 g l^{-1} glucose and 30 g l^{-1}

Table 1. Changes in cell-wall-bound invertase activity (μmol · gFW^{-1} $hour^{-1}$) in cell suspension culture of carrot.

	Treatment	
Days after initiation	30gl^{-1} Sucrose	30gl^{-1} Glucose and 30gl^{-1} Fructose
0	1.27 ± 0.30	1.27 ± 0.30
2	5.50 ± 0.71	3.02 ± 0.45

Each determination repeated at least 3 times (means ± SD).

fructose instead of sucrose, the cell-wall-bound acid invertase activity did not increase to the same extent as that of sucrose treatment (**Table 1**). This result supports that the initiation of cell-wall-bound acid invertase activity requires sucrose and thus occurs only on medium containing sucrose. Straus (1962) showed that the cell-wall-bound invertase activity was higher than that of cytoplasmic enzyme in cultured tissue. Our results also show that once sucrose is taken up by the shaking culture the cell-wall-bound acid invertase activity is initiated and breaks sucrose down to glucose and fructose, thus, decreasing the water potential of the solution (**Figs. 1A, 2 and 3**).

Our work, therefore, shows that changes in water potential of culture solution in carrot are influenced by sucrose breakdown to glucose and fructose, due to cell-wall-bound acid invertase activity.

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