Little information exists concerning the biochemical route of mannitol catabolism in higher plant cells. In this study, the role of mannitol catabolism was investigated. Suspension cultures of celery (Apium graveolens L. var dulce [Mill.] Pers.) were successfully grown on nutrient media with either mannitol, mannose, or sucrose as the sole carbon source. Cell cultures grown on any of the three carbon sources did not differ in relative growth rate, as measured by packed cell volume, but differed drastically in internal carbohydrate concentration. Mannitol-grown cells contained high concentrations of mannitol and extremely low concentrations of sucrose, fructose, glucose, and mannose. Sucrose-grown cells had high concentrations of sucrose early in the growth cycle and contained a substantial hexose pool. Mannose-grown cells had a high mannose concentration early in the cycle, which decreased during the growth cycle, whereas their internal sucrose concentrations remained relatively constant during the entire growth cycle. Celery suspension cultures on all three carbon substrates contained an NAD-dependent MDH. Throughout the growth cycle, MDH activity was 2- to 4-fold higher in mannitol-grown cells compared with sucrose- or mannose-grown cells, which did not contain detectable levels of mannitol, indicating that MDH functions predominantly in an oxidative capacity in situ. The MDH activity observed in celery cells was 3-fold higher than the minimum amount required to account for the observed rate of mannitol utilization from the media. Cultures transferred from mannitol to mannose underwent a decrease in MDH activity over a period of days, and transfer from mannose to mannitol resulted in an increase in MDH activity. These data provide strong evidence that MDH plays an important role in mannitol utilization in celery suspension cultures.

In species such as celery (Apium graveolens L. var dulce [Mill.] Pers.), white-ash (Fraxinus americana L.), common lilac (Syringa vulgaris L.), and European privet (Ligustrum vulgare L.), mannitol is a major photosynthetic product that is transported in the phloem and can accumulate to high concentrations in various tissues (Trip et al., 1963; Keller and Matile, 1989; Davis and Loescher, 1990; Loescher et al., 1992; Stoop and Pharr, 1993). Although the physiological roles of mannitol in higher plants are still unclear, it has been proposed that mannitol may confer beneficial traits. Celery has high photosynthetic rates, as high as 38 μmol CO₂ m⁻² leaf s⁻¹ at light saturation, and this has been attributed to the extra chloroplastic redox turnover in the synthesis of mannitol (Rumpho et al., 1983; Fox et al., 1986). Celery is also highly tolerant of salt in the root environment, which has been attributed to the function of mannitol as a compatible solute (Everard et al., 1992; Stoop and Pharr, 1993, 1994). Recent experiments with tobacco that has been genetically engineered to synthesize mannitol through the introduction of the enzyme NAD-dependent mannitol-1-P dehydrogenase from Escherichia coli (Tarczynski et al., 1992) demonstrated increased salt tolerance as a result of the transformation (Tarczynski et al., 1993).

The pathway of mannitol biosynthesis in photosynthetic celery leaves has been established (Rumpho et al., 1983; Loescher et al., 1992). Mannitol is synthesized in the cytoplasm of leaf mesophyll cells from dihydroxyacetone-P that is exported from the chloroplasts by the Pi translocator. The unique enzyme in this biosynthetic pathway is M6PR, which catalyzes the NADPH-dependent reduction of Man-6-P to mannitol-1-P. The M6PR functions primarily in a synthetic manner, with only very low rates of mannitol-1-P oxidation observed (Loescher et al., 1992). M6PR is distinctly different from mannitol-metabolizing enzymes in lower organisms, all of which can be characterized as 2-oxidoreductases, catalyzing the interconversion of Fru or Fru-6-P to mannitol-1-P. Many of the mannitol dehydrogenases in lower organisms can function in both directions, e.g. mannitol oxidation or Fru reduction, depending on the organism and growth conditions (Lewis and Smith, 1967; Lewis, 1984).

Little information exists concerning mannitol catabolism in higher plants. Mannitol utilization in vascular plants has been shown to occur at low rates in suspension cultures of Monterey pine and carrot (Thompson et al., 1986), as well as at higher rates in leaves of white ash, lilac, and celery (Trip et al., 1964; Fellman and Loescher, 1987). However, it is not known to what extent or by what enzymic route mannitol is assimilated in sink tissues of higher plants. Recently, an
NAD-dependent MDH was discovered and partially purified from celeriac root tips (Stoop and Pharr, 1992). The enzyme was also present in celery sink tissue, where it catalyzes the oxidation of mannitol to Man, which in turn can be phosphorylated to Man-6-P, converted to Fru-6-P, and enter central metabolism (Stoop and Pharr, 1994). Thus, higher plants differ in both mannitol biosynthesis and mannitol catabolism from lower organisms.

To further assess the potential physiological importance of the recently discovered MDH in higher plants, the growth of celery cells on different carbon sources was characterized. Heterotrophic plant tissue cultures of apple (Chong and Taper, 1974; Negm and Loescher, 1979) and cucumber (Gross et al., 1981) have been useful in defining the routes of sorbitol and raffinose saccharide catabolism, respectively. The objectives of this study were (a) to determine if and to what extent mannitol, the substrate of the MDH-catalyzed reaction, and Man, the mannitol oxidation product, could support growth of celery cells in culture; and (b) to determine if mannitol utilization was related to the MDH activity.

**MATERIALS AND METHODS**

**Seed and Plant Material**

Celery seed (Apium graveolens L. var dulce [Mill.] Pers., breeding line CE201) obtained from DNA-Plant Technology Corp. (Cinnaminson, NJ) was surface sterilized in an 8% (v/v) Clorox (5.25% sodium hypochlorite) solution for 20 min, followed by three rinses in sterile, deionized water. Seeds were aseptically transferred in sterile 7.5 × 7.5 × 10 cm polypropylene vessels (Magenta, Chicago) containing half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) at pH 6.7, solidified with 2% (w/v) Biterate agar (Difco Laboratories, Detroit, MI). The polypropylene vessels were placed in an environmental chamber with 8/16 h day/night at 25°C.

**Initiation and Maintenance of Celery Suspension Cultures**

Celery suspension cultures were initiated from hypocotyls of aseptically grown celery plants. Hypocotyls were removed from plantlets 25 d after germination and were surface sterilized in 8% (v/v) Clorox with slight agitation for 20 min. Hypocotyls were turned over while in Clorox solution after 10 min. This was followed by three rinses in sterile, deionized water (3–5 min per rinse). Hypocotyls were transferred onto sterile Petri dishes (100 × 15 mm) where bleached ends were removed. Individual hypocotyls were cut into 5-mm segments and transferred into 250-mL wide-necked Erlenmeyer flasks containing the induction medium of 50 mL of Murashige and Skoog basal medium (inorganic salts and vitamins, Murashige and Skoog, 1962) supplemented with 2.25 μM 2,4-D, 1.8 mg/L of kinetin, and 90 mM Suc, and adjusted to pH 5.7 before autoclaving. Cultures were held at room temperature on a gyratory shaker at 100 rpm under constant 10 to 60 μmol m⁻² s⁻¹ PAR. After 25 d, cells were transferred into 500-mL Erlenmeyer flasks containing the same induction medium. Cells were subsequently transferred to induction medium containing mannitol (180 mM), Man (180 mM), or Suc (90 mM) as the exclusive carbon source; the cultures were subcultured every 20 d. Because cells can hydrolyze external Suc to Fru and Glc rather quickly, a 90 mM Suc concentration was chosen that represented a theoretical equivalent of 180 mM hexoses. Each treatment was replicated four times.

**Growth Determination**

Growth curves were established by plotting PCV over days after transfer. PCV measures the volume of cells in a given culture volume by collecting the cells by centrifugation. Cell cultures were grown for 19 to 20 d before being subcultured. The volume of cells to be subcultured was determined so that the PCV at d 0 was close to 0.05 mL cell/mL culture. PCV was measured at several time intervals during the growth cycle by aseptically sampling 5 or 10 mL of culture and centrifuging it for 2 min at 1000 rpm in a table-top centrifuge (IEC HN-SII, Needham, MA). PCV was measured and the supernatant was decanted and kept for analysis of media carbohydrates. Cells were rinsed by adding 10:1 (v/v) deionized water, stirring the mixture, and centrifuging it at 1000 rpm for 2 min. The resulting supernatant was discarded and the cells were used for carbohydrate and enzyme analysis. The RGR was determined by plotting the PCV over time, resulting in an exponential growth curve of which the exponent represents the RGR (mL cell d⁻¹) × mL⁻¹ cell).

**Carbohydrate Analysis**

Ethanolic extractions of cells from each of the samples were used for measurements of Suc, hexose sugars, and mannitol. Elapsed time between aseptic cell harvest and ethanolic extraction varied between 30 min and 1 h. The procedures for ethanolic extraction were followed as described by Hubbard et al. (1990). Carbohydrate content of the cells was determined on an HPLC system using a BC-100 Ca²⁺ column (Benson, Reno, NV) operated at 75°C with deionized, degassed water as the C₄₅ eluent. The column was preceded by a Waters Bondapak C₈/Corsaril guard and a set of anion and cation de-ashing cartridges (Bio-Rad). Media carbohydrates were analyzed on the same HPLC system. When the concentration of media carbohydrates (y axis) was regressed against the volume of cells in the culture (x axis), a linear relationship was obtained, of which the slope (dy/dx) represents the amount of carbohydrates used (mg) to produce 1 mL of cells. Slopes were statistically compared using the estimates of slopes and their se values obtained from the analysis of variance model (SAS, Cary, NC).

**Enzyme Extraction and Assays**

Celery suspension cells were harvested as described above and ground in a chilled mortar using a 1:4 (v/v) tissue:buffer ratio. The extraction buffer contained 50 mM Mops (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, 5 mM DTT, and 1% Triton X-100. Homogenates were transferred to microcentrifuge tubes and centrifuged at 10,000g for 1 min and desalted by centrifugal filtration on a Sephadex G-25-50 column equilibrated with 50 mM Mops-NaOH (pH 7.5), 5 mM MgCl₂, and 5 mM DTT prior to assay for MDH activity. Samples were stored on ice. MDH activity was assayed by monitoring the reduction of
NAD spectrophotometrically at 340 nm. Assays were conducted at room temperature (25°C) in a total volume of 1 mL. The reaction mixture contained 100 mM Bis-Tris propane (pH 9.0), 2 mM NAD⁺, enzyme extract, and 150 mM D-mannitol. The reactions were initiated with mannitol. One unit of activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol NAD⁺/h. Initial rates of oxidation were used to calculate MDH activity. Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

The product of the oxidation of mannitol catalyzed by MDH from celery suspension cultures was purified and identified as described for celeriac root tips by Stoop and Pharr (1992).

RESULTS

RGR

Celery cell-suspension cultures were successfully established and they grew on mannitol, Man, or Suc as the sole carbon source. Regardless of the carbon source, cells grew exponentially during the entire experimental period (Fig. 1). Growth curves similar to that shown in Figure 1 were obtained during the 12-month period of this study. The RGR of cells grown on mannitol or Man did not statistically differ from the RGR of cells grown on Suc (Table I).

Media Depletion and Internal Carbohydrate Concentrations

Cells grown on mannitol differed from Suc-grown cells in that they depleted the external carbon source without converting it to hexose prior to uptake (Fig. 2A). Suc-grown cells depleted Suc but also hydrolyzed Suc to Fru and Glc, which accumulated in the media through d 13 (Fig. 2B). Thereafter, external hexoses and Suc were further depleted to support cell growth. Man-grown cells responded similarly to mannitol-grown cells by depleting the external Man without converting it to other hexoses prior to uptake (Fig. 2C).

Cells grown on mannitol, Man, or Suc were able to take up external carbohydrate rather quickly, since internal carbohydrate pools, determined 30 min to 1 h after transfer to the new media, were high on d 0 compared with their levels prior to cell transfer (Fig. 2, D–F). Cells from the end of the previous cycle had carbohydrate levels similar to those at d 19 (Fig. 2, D–F). Celery suspension cells grown on different carbon sources differed drastically in internal soluble carbohydrate concentrations. The internal soluble carbohydrate pool of mannitol-grown cells consisted almost entirely of mannitol and was extremely low in hexose (Glc and Fru) and in Suc (Fig. 2D). Man was present at about 5- to 10-fold lower concentration than Glc or Fru in mannitol-grown cells (data not shown). Early in the growth cycle the internal soluble carbohydrate pool of Suc-grown cells consisted mainly of Suc (Fig. 2E). Toward the end of the growth cycle Suc concentrations dropped substantially, but a minimum Suc pool remained (± 2 mg/mL). Hexose levels peaked at d 5 but remained at least 2-fold lower than Suc levels, and Fru was always higher in concentration than Glc (Fig. 2E). Man-grown cells had a high initial Man pool, which decreased during the growth cycle, whereas their internal Suc level remained relatively constant over the growth cycle (Fig. 2F). The hexose concentration of Man-grown cells was higher than that of mannitol-grown cells and lower than that of Suc-grown cells. Neither Suc- nor Man-grown cells contained detectable mannitol. In all cell cultures, internal carbohydrate concentrations seemed to be related to media carbohydrate concentrations as internal carbohydrate pools decreased with decreasing external pools (Fig. 2).

When cells grown for several months on Man, Suc, or mannitol were transferred to a solely mannitol-containing medium, the internal carbohydrate pool of cells from all treatments was similar to that of the mannitol-grown culture depicted in Figure 2D. When cells adapted to growth on any one of the three carbon sources were transferred to either Suc or Man medium, internal carbohydrate pools were similar to that of Suc or Man-grown cells depicted in Figure 2, E or F, respectively. Therefore, the internal carbohydrate pool of cells grown in suspension culture is affected by the current carbon source in the media much more strongly than by the external carbon source used in previous growth cycles (data not shown).

When cells adapted to growth on Suc or mannitol were grown in a mixture of Suc and mannitol (50:50, w/w), no significant difference in RGR or in the amount of carbohydrate used (mg) per volume of cells produced (mL) could be observed when compared with cells grown solely on mannitol.

Table I. RGR ([ml cell d⁻¹]-ml⁻¹ cell) of celery suspension cells grown on three different carbon sources.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>RGR (ml cell d⁻¹)-ml⁻¹ cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>0.110 ± 0.004a</td>
</tr>
<tr>
<td>Man</td>
<td>0.107 ± 0.003a</td>
</tr>
<tr>
<td>Suc</td>
<td>0.102 ± 0.006a</td>
</tr>
</tbody>
</table>

Data represent mean ± se of five different growth cycles (four replications per cycle). Means with the same letter are not significantly different at α = 0.05.
Figure 2. Changes in concentration of the media carbohydrates of celery suspension cultures grown on mannitol (A), Suc (B), or Man (C) as the sole carbon source. Suc-grown cells hydrolyzed external Suc (V) to Fru (O) and Glc (A), with (+) representing the sum of these external sugars (B). Internal mannitol (○), Suc (V), Man (□), Glc (▲), and Fru (○) concentrations of celery suspension cultures grown on mannitol (D), Suc (E), or Man (F) as the sole carbon source. Mannitol was not detected in Suc- or Man-grown cells (E, F). Data represent mean ± se of four replications.

or Suc (Table II). However, early in the growth cycle (up to d 8) mannitol- and Suc-adapted cells differed in the relative amount of mannitol and sugar (sum of external Suc, Fru, and Glc) depletion (Fig. 3). Mannitol-adapted cells depleted mannitol and Suc in about equal amounts starting immediately after cell transfer (Fig. 3A). In contrast, the Suc-adapted cells preferentially used Suc above mannitol, especially during the first 4 d (Fig. 3B). When the amount of mannitol used (mg) to produce 1 mL of cells and the amount of sugar used to produce 1 mL of cells was determined by regression analysis of mannitol or sugar concentration in the media versus cell volume, using data up to d 8, a significant difference could be observed between mannitol- and Suc-adapted cells (Table II). Suc-adapted cells used more sugar and less mannitol compared with the mannitol-adapted cells. After 8 d the two cell lines did not differ significantly (data not shown), which suggested that early in the growth cycle mannitol-adapted cells had the capacity to utilize mannitol together with Suc.

Table II. RGR and carbohydrate used of celery suspension cells grown on mixed carbon sources

<table>
<thead>
<tr>
<th>Carbon Source Prior to Transfer</th>
<th>Carbon Source during Growth</th>
<th>RGR 10 d⁻¹</th>
<th>Carbohydrate Used¹</th>
<th>First 8 d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg mL⁻¹ cell</td>
<td>mg mL⁻¹ cell</td>
<td>Mannitol Used</td>
</tr>
<tr>
<td>Suc</td>
<td>Suc</td>
<td>0.101 ± 0.010a</td>
<td>502 ± 32a</td>
<td>502 ± 32a</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Mannitol</td>
<td>0.105 ± 0.004a</td>
<td>445 ± 25a</td>
<td>445 ± 25a</td>
</tr>
<tr>
<td>Suc</td>
<td>Mannitol + Suc</td>
<td>0.103 ± 0.003a</td>
<td>436 ± 23a</td>
<td>395 ± 39a</td>
</tr>
<tr>
<td>Mannitol</td>
<td>Mannitol + Suc</td>
<td>0.106 ± 0.003a</td>
<td>504 ± 23a</td>
<td>243 ± 27b</td>
</tr>
</tbody>
</table>

¹ Carbohydrate = Suc + Glc + Fru + mannitol.  
² Sugar = Suc + Glc + Fru.

Data represent mean ± se of six replications. Means with the same letters within the same column are not significantly different at α = 0.07.
Mannitol 1-Oxidoreductase in Celery Suspension Cultures

Figure 3. Depletion of mannitol and sugars (Suc + Fru + Glc) from the medium of mannitol-adapted cells (A) and Suc-adapted cells (B) transferred to a medium containing both Suc and mannitol (50:50, w/w) at d 0. Data represent mean ± se of four replications.

whereas Suc-adapted cells were more restricted to Suc utilization. Later in the growth cycle, Suc-adapted cells utilized mannitol as well as Suc, indicating the possibility for a requirement of an induction period to provide the mechanism that enables those cells to utilize mannitol.

MDH

Celery suspension cultures grown on mannitol, Man, and Suc contained an NAD-dependent MDH. The product of the oxidation of mannitol, catalyzed by the MDH, was shown to be exclusively Man by protocols similar to those published for the celeriac root enzyme (Stoop and Pharr, 1992). At any time during the growth cycle, MDH activity was 2- to 3-fold higher in mannitol-grown cells compared with Suc-grown cells, whereas Man-grown cells had very low to undetectable MDH activities (Fig. 4). Similar relationships were observed for specific activities (µmol h⁻¹ mg⁻¹ protein) of MDH (data not shown). The MDH activity peaked 4 to 7 d after transfer and decreased during the rest of the culture cycle (Fig. 4).

The question of whether the cells contained adequate MDH activity to account for the observed rate of depletion of mannitol from the culture medium was addressed, and data relevant are shown in Figure 5. The rate of depletion of mannitol from the media of individual flasks for discrete 4-d time intervals over the culture cycle was plotted as a function of the mean MDH activity during each interval (Fig. 5). The regression analysis indicated that the activity of MDH was positively correlated with the rate of mannitol depletion. Furthermore, the slope of the regression indicated that cells contained about a 3-fold excess of the minimum amount of enzyme activity necessary to account for the observed rate of mannitol depletion.

When mannitol-adapted cells were transferred to media containing Suc or Man as the sole carbon source, MDH activity of the developing culture was suppressed compared with the MDH activity of cells grown continuously on mannitol (Fig. 6A). This suggests that the current carbon source strongly influences the MDH activity of the cells. Man- or Suc-grown cells transferred to mannitol media underwent an increase in MDH activity over a period of several days (Fig. 6, B and C). No major differences were observed when Man-grown cells were transferred to Suc media and vice versa.

DISCUSSION

The results demonstrate that celery suspension cells are capable of utilizing Suc, mannitol, or Man (the oxidation product of mannitol) as the sole carbon source for growth. Cells grown on mannitol or Man had growth rates similar to cells grown on the more commonly used carbon source, Suc. Celery cells are different from those of many species in that mannitol and Man are rapidly taken up by the cells and
Prunus persica

It is probable that the ability of cultured cells to accumulate and is believed to be nearly metabolically inert because it is very slowly taken up by a wide range of plant cells and translocate this polyol to sinks. In many plant species, Man is readily phosphorylated but not further metabolized due to the low activity of or the absence of PMI, a key enzyme that allows Man to enter the pool of glycolytic intermediates (Herold and Lewis, 1977). In these species, Man feeding results in lowered endogenous Pi and decreased ATP availability, which induces inhibitory effects on various metabolic processes (Herold and Lewis, 1977). Celery differs from these species in that it contains very high PMI activities in source and sink tissues (Stoop and Pharr, 1994). A high PMI activity (162 \( \mu \text{mol h}^{-1} \text{mL}^{-1} \text{cell} \)) was also observed in celery suspension cultures.

The composition of the internal carbohydrate pool of celery cells was strongly dependent on the carbon source supplied in the media. Mannitol-grown cells contained high levels of mannitol and extremely low levels of Suc, Man, and hexoses. Suc-grown cells had high levels of Suc early in the growth cycle and substantial hexose pools that peaked at mid-cycle. Man-grown cells accumulated primarily Man and Suc and contained low concentrations of Fru and Glc. This drastic effect of exogenous carbon on internal carbohydrate pools differs from cucumber (Gross et al., 1981) or sugarcane cell cultures (Maretzki and Thom, 1978), where, regardless of carbon source, cells predominantly accumulate Suc, Glc, and Fru, except for Gal-grown cultures, which accumulate Gal in addition to other soluble sugars (Maretzki and Thom, 1978). Internal and media carbohydrate concentrations decreased in a correlated manner throughout the culture cycle, indicating that external carbohydrate concentration might affect the magnitude of the internal carbohydrate concentration.

Data presented here support the hypothesis that mannitol utilization may occur by the NAD-dependent MDH activity. The MDH activity observed in celery cells was 3-fold higher than the minimum amount required to account for the observed rate of mannitol utilization from the media. Suc- and Man-grown cells did not contain detectable levels of mannitol, indicating that MDH functions predominantly in an oxidative capacity in situ. Cells transferred from mannitol to Man underwent a decrease in MDH activity over a period of days, and transfer from Man to mannitol resulted in an increase in MDH activity. This is, to some extent, analogous to substrate induction and repression of mannitol-1-P dehydrogenase in bacteria (Martinez De Drets and Arias, 1970). However, in bacteria the variation in enzyme activity in response to substrates is much greater than in these celery cells. The decrease in MDH activity of mannitol-adapted cells grown on Man and the increase in MDH activity of Man-adapted cells grown on mannitol suggest that the observed MDH activity might be caused by a direct or indirect effect of the current carbon source. Alternatively, these changes in MDH activity could be from selection for specific biochemical cell type from a heterogeneous mixture of cells differing in their ability to metabolize mannitol.

MDH might represent the initial step of the enzymic route of mannitol assimilation in sink tissues by converting mannitol to Man. Man can be readily phosphorylated and further metabolized by PMI to Fru-6-P, which can enter central metabolism. However, these data cannot answer the question of whether an additional route of mannitol catabolism exists in these cells. Such a question may be highly relevant when considering sink metabolism, because multiple invertases, as well as Suc synthases, exist simultaneously in sink tissues of...
Suc-translocating plants (Ho, 1988; Sung et al., 1988), and all of these enzymes may operate simultaneously in some sinks (Hubbard et al., 1989). Multiple α-galactosidases as well as the Suc-degrading enzymes exist in the sinks of raffinose saccharide-translocating plants (Pharr and Sox, 1984), whereas sorbitol dehydrogenase and sorbitol oxidase have been reported in sinks of sorbitol-translocating plants (Yamaki and Ishikawa, 1986; Moriguchi et al., 1990).

The ability of celery cells to grow on mannitol and its oxidation product Man as the sole carbon source provides a useful system to further study mannitol catabolism in vitro. Furthermore, it may prove useful that Suc-grown cells do not contain an internal mannitol pool whereas mannitol-grown cells do contain an internal pool of mannitol. This characteristic is currently being used to study the role of internal mannitol in salt tolerance at the cellular level.

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**LITERATURE CITED**


