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EFFECT OF MANNITOL AND GLUCOSE-INDUCED OSMOTIC STRESS ON GROWTH, WATER RELATIONS, AND SOLUTE COMPOSITION OF CELL SUSPENSION CULTURES OF POPLAR (*POPULUS DELTOIDES* VAR. *OCCIDENTALIS*) IN RELATION TO ANTHOCYANIN ACCUMULATION

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SUMMARY

A cell suspension culture of poplar (*Populus deltoides* (Marsh.) Bartr. var. *occidentalis* Rydb.), accumulating the anthocyanin pigment, cyanidin 3-glucoside, in the lag phase of culture growth, was subjected to osmotic stress with glucose and mannitol. Osmotic stress treatments resulted in growth suppression and higher anthocyanin accumulation compared with unstressed cells. Both an increase in the proportion of pigmented cells and an increase in the concentration of anthocyanin in the pigmented cells were responsible for high anthocyanin content of cultured cells subjected to osmotic stress. The osmotic stress induced by glucose suppressed growth more than that by mannitol and produced higher anthocyanin levels. Only small amounts of [U-^{14}C]mannitol were taken up and metabolized by the cells. Stressed cells accumulated sugars and free amino acids to a different extent resulting in altered cell sugar-to-amino acid ratios. The accumulation of osmotically active solutes and cell growth suppression may both be responsible for the accumulation of anthocyanin in stressed cells.

Key words: anthocyanin accumulation; free amino acids; mannitol metabolism; osmotic potential; poplar cell suspension; soluble sugars; water potential.

INTRODUCTION

The production of secondary metabolites in cell cultures is influenced by various culture conditions including osmotic stress (Mantel and Smith, 1983). However, the exact mechanism of the osmotically induced accumulation of secondary products remains unclear. Osmotic stress in cell culture can be imposed by the addition of salts or organic solutes to the culture medium. In addition to the osmotic effect, these solutes can affect cells in a variety of ways. A high concentration of sugars in the medium is known to increase secondary metabolite production in cell cultures (van der Heijden et al., 1989). This effect of high sugar concentration in the medium could be due either to the osmotic effect or to the role of sugars as a carbon source. To separate the osmotic effect of sugars from their role as a carbon source, mannitol is often added to the culture medium and the resultant effect on growth and secondary metabolism is attributed to the osmotic effect (Do and Cormier, 1990). However, mannitol can be absorbed by certain cell cultures and metabolized (Thompson et al., 1986). Mannitol is also considered to be a compatible solute which can accumulate in high concentrations in plant cells without severe inhibition of cell processes (Borowitzka, 1981). Therefore, the addition of mannitol to the cell culture medium might influence cell growth and secondary metabolism in a different way than sugars.

The mechanism of osmotic stress-induced secondary metabolite production may be different in cell cultures producing the metabolites during the stationary phase from those producing the metabolites during the lag phase of culture growth. It has been shown that the composition of solutes in the cells changes greatly from lag to stationary phase (Lindsey and Yeoman, 1985). However, the composition of soluble carbohydrate in the cells osmotically stressed with sugars or sugar alcohols has apparently never been studied in relation to secondary metabolite production. Such information is necessary to understand the role of these solutes in secondary metabolite production. An inverse relationship between growth and secondary metabolite accumulation seems to be a common feature of plant cell cultures (Mantel and Smith, 1983).

In the present study we used a cell culture of poplar which accumulated anthocyanins during the lag phase of growth when the source of carbon was not limiting to growth. This approach permitted us to compare the osmotic effects of solutes differing in the degree to which they are metabolized under non-limiting conditions of carbon source. The principal objective of the present study was to examine the mechanisms of anthocyanin accumulation in osmotically stressed cells. We compare the effects of osmotic stress in-

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duced by mannitol and glucose on anthocyanin levels, growth, water relations, and solute composition in cell suspension cultures of poplar (*Populus deltoides* var. *occidentalis*).

Materials and Methods

Culture initiation and maintenance. Young leaves of *Populus deltoides* (Marsh.) Bartr. var. *occidentalis* Rydb. were obtained from the Devonian Botanical Gardens, Devon, Alberta. Sterile leaf explants, about 0.5 cm^2 in size were used to initiate callus on Murashige and Skoog (1962) agar medium (MS) containing 3% sucrose and 5 μM each of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. Those calli that developed red pigments on their outer surfaces when exposed to light were transferred to 250-ml flasks containing 100 ml MS liquid medium. The MS liquid medium contained 3% sucrose and 2 μM 2,4-D and 1 μM kinetin, the growth regulator concentrations that were found to be optimal for anthocyanin production in a hybrid poplar cell suspension culture (Matsumoto et al., 1971). The cultures were kept agitated on a rotary shaker at 120 rpm at room temperature under continuous wide-spectrum fluorescent light (Gro and Sho, model F40/GS/W9) at a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The soft, red-pigmented part of the callus formed a suspension of small aggregates whereas hard and green inner portions were removed after 10 days. The cultures were transferred to fresh medium every 10 to 15 days.

Anthocyanin identification. Anthocyanin was extracted from cells with 1% HCl in methanol. The extract was filtered and concentrated in vacuo at 30° C for use in paper chromatography. For spectral analysis the extract was washed with ethyl acetate and the aqueous fraction was concentrated as above. The concentrate was further purified by preparative paper chromatography using butanol-acetic acid-water (4:1:5) as the solvent system (Harborne, 1984). The anthocyanin was identified by paper chromatography according to the procedure given by Harborne (1984). Standards of cyanidin 3-glucoside and cyanidin 3,5-diglucoside were obtained from rose petals.

The anthocyanin and the sugar were identified by paper chromatography after acid hydrolysis (Harborne, 1984). Cyanidin, delphinidin, and pelargonidin standards were obtained from rose petals, outer pericarp of egg plant fruit, and pomegranate fruit, respectively. The number of sugar molecules was identified by controlled hydrolysis and the position of the sugar molecule by peroxide hydrolysis (Francis, 1982).

Measurements of osmotic and water potentials. The osmotic potential of the medium and water and osmotic potential of the cells were determined using a HR-33T Wescor (Logan, Utah) microvoltmeter equipped with a triple psychrometer chamber (Dixon Instruments Inc., Guelph, Ontario), in the dew point mode. Cell water potential was determined after filtering and spin drying the cells at 200 *g* for 5 min to remove surface moisture (Fallon and Phillips, 1988). For the determination of cell osmotic potential, the cells were wrapped in aluminum foil and frozen overnight at −30° C before thawing.

Osmotic stress treatments. Standard curves of glucose and mannitol concentrations in MS medium vs. osmotic potential were obtained by experimental determinations of osmotic potentials. An osmotic potential of −0.6 MPa was chosen as control (105 mM glucose), and −1.1 MPa (326 mM glucose or 210 mM mannitol) and −1.6 MPa (547 mM glucose or 450 mM mannitol) were chosen for glucose and mannitol stress treatments, after initial experimentation.

Cells from 12- to 15-day-old cultures (linear phase of culture growth) at an inoculum density of 6.5% packed cell volume (PCV) were used in the experiment. Aliquots of the cell suspension were taken every 3 days from the time of culture inoculation up to 12 days. PCV, cell number, osmotic potential of media, and water potential, osmotic potential, anthocyanin, total soluble sugar, and amino acid content of the cells were determined. The sugar composition of the cells was determined 12 days after culture inoculation. Each osmotic stress treatment consisted of three replications.

Growth measurements. PCV was determined by centrifuging aliquots of cell suspension at 200 *g* for 5 min (Dixon, 1985). Cell dry weight was determined after filtering, washing, and drying the cells for 12 h at 60° C (Dixon, 1985). Cell number and number of pigmented cells were determined for control and −1.6 MPa stress levels of glucose and mannitol by taking aliquots of fine cell suspension and counting the pigmented and nonpigmented cells using a hemacytometer. The amount of anthocyanin per packed pigmented cell volume was used as an indicator of anthocyanin concentration in the cells, and was calculated by dividing anthocyanin per milliliter of culture by packed pigmented cell volume per milliliter of culture. The packed pigmented cell volume was calculated from packed cell volume per milliliter culture and pigmented cell number per milliliter culture. Anthocyanin per pigmented cell was calculated by dividing anthocyanin per milliliter culture volume by the number of pigmented cells in 1 ml of culture.

Quantification of anthocyanin content of cells. The anthocyanin was extracted from the cells with 1% HCl in methanol, and the absorbance of the anthocyanin extract was measured at 525 nm. The molar extinction coefficient of cyanidin 3-glucoside was used to calculate the anthocyanin content (Francis, 1982).

Determination of soluble sugars and free amino acids. The harvested cells were washed in deionized water and freeze-dried. Five milligram samples of freeze-dried cells were ground in a glass mortar, and soluble sugars and free amino acids were extracted with 5 ml hot 80% ethanol for 30 min. Extracts were filtered and the residue was extracted twice in 80% ethanol. Filtrates were combined and evaporated to dryness in vacuo at 40° C. Total soluble sugars were quantified as β-D-glucose equivalents by the anthrone method (Ashwell, 1957). The total free amino acids were quantified as L-leucine equivalents by the modified ninhydrin method (Rosen, 1957).

For determination of the soluble carbohydrate composition, extracts from 10-mg, freeze-dried cells were prepared as above and the carbohydrates were purified using a 0.5 × 7.0-cm Dowex 50W (200 mesh) cation exchange column (H^+ form) followed by 0.5 × 7.0-cm Dowex 50W (200 mesh) anion exchange column (Cl^- form) as described by Zwiazek and Shay (1988). After overnight derivatization, trimethylsilyl (TMS) ethers of carbohydrates were analyzed using a Hewlett Packard 5890 gas chromatograph equipped with a model 3396A electronic integrator (Hewlett Packard, Mississagua, ON). Samples were separated on a 30-m-long, 0.25-mm i.d. fused silica DB-5 capillary column, 0.25-μm film thickness (J & W Scientific, Folsom, CA). Conditions during the run were as follows: Initial oven temperature 180° C, increased to 210° C at the rate of 2° C $\cdot$ min^{-1}; followed by 210° to 250° C at the rate of 5° C $\cdot$ min^{-1} and held for 10 min at 250° C; injector and flame ionization detector temperature 300° C; 50:1 split injection; carrier gas, (helium) linear flow rate 25 cm $\cdot$ s^{-1}. Carbohydrates were identified by co-chromatography with authentic carbohydrate standards obtained from Sigma (St. Louis, MO) and were quantified using sorbitol as an internal standard.

Determination of mannitol metabolism. Aliquots from 12-day-old cultures of −1.1 and −1.6 MPa mannitol stress treatments were transferred to 25-ml Erlenmeyer flasks under aseptic conditions. [U-^{14}C]mannitol was added to the cultures to obtain a final specific activity of 20 Bq $\cdot$ $mmol^{-1}$. A filter paper saturated with 25% KOH serving as a carbon dioxide trap was hung at the top of each flask and the flasks were sealed with a serum cap and incubated for 14 h in an orbital shaker at 120 rpm. At the end of incubation, the filter paper was placed in a scintillation vial containing the Scintiverse E scintillation cocktail (Fischer Scientific Company, Edmonton, AB). The radioactivity was measured using a Minaxi B Tricarb 4000 series liquid scintillation counter.

Statistical analysis. The data were statistically analyzed by the analysis of variance (ANOVA) procedure, and least significant difference test ($P \leq 0.05$) was used for mean comparisons.

Results

Spectral comparison and paper chromatography of anthocyanin released from the anthocyanin accumulating in cultured cells identified the aglycone as cyanidin. Peroxide hydrolysis indicated that the attachment of the sugar molecule to the aglycone was in the C-3 position. Finally, the anthocyanin was identified as cyanidin 3-glucoside by paper chromatography.

Culture growth stage and anthocyanin accumulation. The growth pattern of the culture followed a sigmoidal curve as indicated by %PCV (Fig. 1). Anthocyanin per unit PCV continued to increase until the end of lag phase and early exponential phase, after which it decreased.

Growth and anthocyanin accumulation. Osmotic stress treatments caused an increase in the duration of the lag phase by 3 to 6

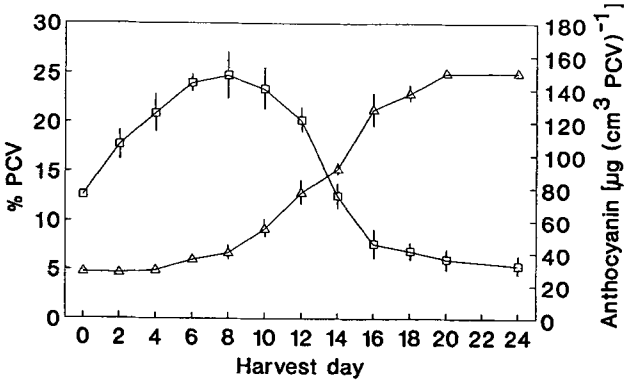

FIG. 1. Changes in cell growth and anthocyanin content of poplar cell suspensions at different days after culture inoculation. Each point is the mean of three replicates. *Error bars* represent ±SE. *Open triangles*, %PCV; *open squares*, Anthocyanin.

days and significantly decreased cell growth as indicated by the decrease in percent packed cell volume (Fig. 2 *a*). The difference in growth between the control and stress treatments increased until the last harvest day. Higher levels of each stress treatment caused greater growth suppression. However, both levels of glucose-induced osmotic stress caused greater growth suppression than the same levels of osmotic stress induced by mannitol (Fig. 2 *a*). The rate of growth after the lag phase was lower in both mannitol stress treatments compared with the same level of glucose stress treatment. For the first 3-day period after the lag phase, culture growth rate in −1.1 MPa mannitol stress treatment was 1.33 cm^3 PCV · day^{-1}, whereas it was 0.56 cm^3 PCV · day^{-1} for the same level of glucose stress treatment.

Cell number followed a similar trend to that of the packed cell volume (Fig. 2 *b*). Cell number increased less with increasing osmotic stress levels, and cell number was greater in mannitol stress treatments compared with the same level of glucose stress treatments (Fig. 2 *b*).

The anthocyanin content per unit packed cell volume (Fig. 2 *c*) increased in all treatments and in control cells with harvest time. The increase continued until the harvest Day 12 in −1.6 MPa glucose treatment and Day 9 in the remaining treatments and in control cells. The anthocyanin content was also significantly higher in stress treatments compared with the control, and the differences between the control and stress treatments became greater with time. Higher stress levels caused a greater increase in anthocyanin content per unit PCV. On Day 12, cells stressed by glucose had significantly more anthocyanin compared with those stressed to the same level by mannitol (Fig. 2 *c*).

Percentage of pigmented cells (Fig. 3 *a*), anthocyanin content per pigmented packed cell volume (Fig. 3 *b*), and anthocyanin content per pigmented cell (Fig. 3 *c*) were determined for the control and −1.6 MPa osmotic stress treatments of glucose and mannitol, to determine the cause for the difference in stress-induced anthocyanin content at the cellular level. On Day 12 the percentage of pigmented cells, anthocyanin content per pigmented PCV, and anthocyanin content per pigmented cell were significantly higher in osmotic stress treatments than in control cells. At that time, mannitol stress at −1.6 MPa produced significantly higher percentage of pigmented cells than the same level of glucose stress (Fig. 3 *a*), whereas anthocyanin per pigmented packed cell volume (Fig. 3 *b*) and anthocyanin per pigmented cell (Fig. 3 *c*) were significantly higher in −1.6 MPa glucose stress treatment compared with the same level of mannitol stress.

Water relations. The osmotic potential of the media increased (became less negative) with harvest time in all treatments. In the control, the osmotic potential increased from −0.6 MPa at Day 0 to −0.267 MPa at Day 12. In the same time, −1.1 MPa glucose treatment and −1.1 MPa mannitol treatment increased to −0.811 and −0.7 MPa, respectively. The difference between these two stress treatments at Day 12 was statistically significant. In −1.6

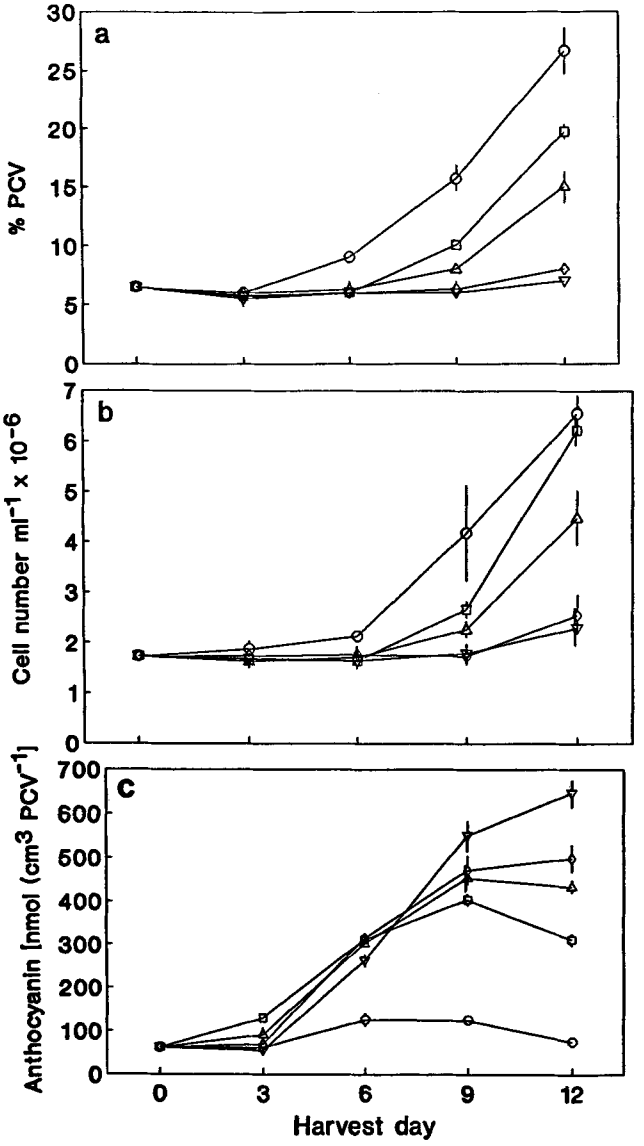

FIG. 2. Effects of osmotic stress with glucose and mannitol on percent packed cell volume (%PCV) (*a*), cell number (*b*) and anthocyanin content (*c*) at different days after culture inoculation in suspension culture of poplar. Each point is the mean of three replicates. *Error bars* represent ±SE. Least significant differences ($P \leq 0.05$) for mean comparison are 1.39 for %PCV (*a*), 0.95 for cell number (*b*), and 41.2 for anthocyanin (*c*). Control (−0.6 MPa); glucose (−1.1 MPa); mannitol (−1.1 MPa); mannitol (−1.6 MPa); glucose (−1.6 MPa).

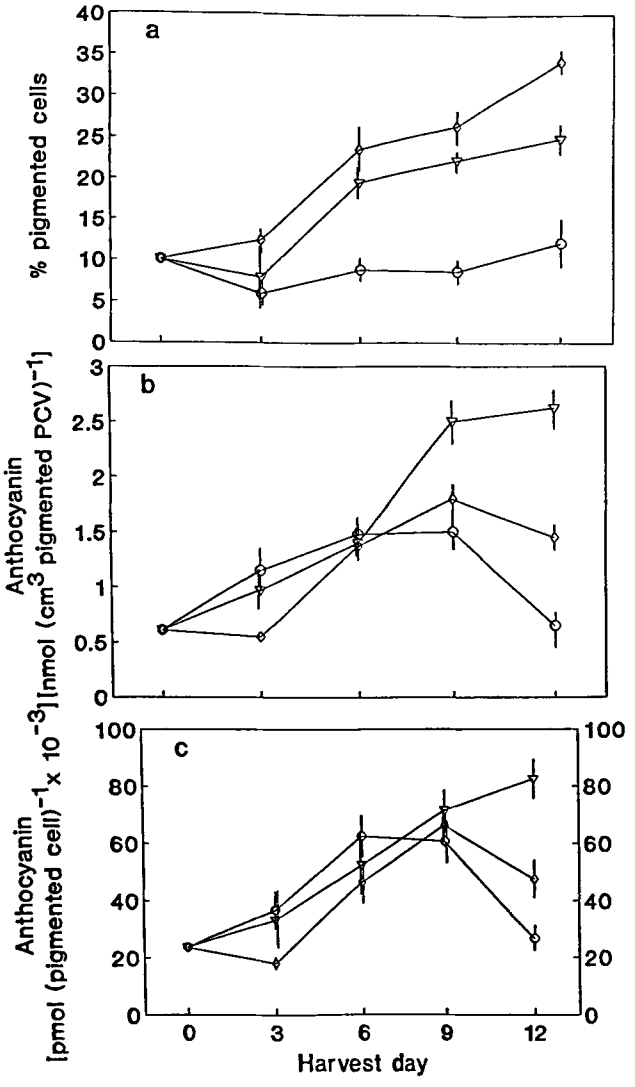

FIG. 3. Percentage of pigmented cells (*a*) and the concentration of anthocyanin measured per packed cell volume (*b*) and pigmented cell (*c*) at different days after culture inoculation in suspension culture of poplar subjected to −1.6 MPa treatments with glucose and mannitol. Each point is the mean of three replicates. *Error bars* represent ±SE. Least significant differences ($P \leq 0.05$) for mean comparison are 6.26 for percent pigmented cells (*a*) 0.51 for anthocyanin per pigmented PCV (*b*), and 20.2 for anthocyanin per pigmented cells (*c*). -⊖- Control (0.6 MPa); -▽- glucose (−1.6 MPa); -◇- mannitol (−1.6 MPa).

MPa glucose treatment and −1.6 MPa mannitol treatment, medium osmotic potentials increased from −1.6 MPa at Day 0 to −1.389 MPa and −1.345 MPa, respectively, at Day 12.

Cell water and osmotic potentials decreased (became more negative) due to osmotic stress treatments (Table 1). Significantly lower cell water and osmotic potentials were observed in all stress treatments compared with the control by Day 3 and the difference was significant until the last harvest day. On the last harvest day, both the water and osmotic potentials of the cells subjected to the initial osmotic potential of −1.1 MPa with mannitol were significantly higher (by 0.313 MPa and 0.337 MPa, respectively) than those subjected to the same level of osmotic stress with glucose.

Soluble sugar and amino acid content of cells. The soluble sugar content of the cells increased on Day 6 in both control and stress treatments (Table 2). Although the soluble sugar content continued to increase until the last harvest day in stress treatments, it decreased on Day 12 in control. Differences in soluble sugar content between control and stress treatments were observed on Day 6, and the difference increased further on Days 9 and 12. On Day 12, cells stressed with glucose at −1.1 MPa had the highest soluble sugar content (4.47 mg · cm^3 PCV^{-1}), whereas those stressed with mannitol at −1.1 MPa had the lowest content (2.82 mg · cm^3 PCV^{-1}).

Free amino acid content of the cells decreased slightly on Day 3 (Table 2). It increased on Day 9 and continued to increase until the last harvest day, in both the control and stress treatments. Significantly higher amino acid content was observed in all the stress treatments compared with control, on Day 12. The −1.1 MPa stress treatments of both mannitol and glucose induced significantly higher amino acid accumulation in the cells than the −1.6 MPa stress treatments, on Day 12. On the same harvest day, cells subjected to osmotic stress with glucose at −1.1 MPa produced significantly higher amino acid levels (2.08 mg · cm^3 PCV^{-1}) than those stressed with mannitol (1.65 mg · cm^3 PCV^{-1}) at the same stress level.

The ratio of soluble sugars to amino acids increased on Day 3 in all stress treatments and remained higher compared with control throughout the harvest time (Table 2). Cells subjected to osmotic stress with glucose at −1.1 MPa showed a significantly higher ratio of soluble sugar to amino acids than those subjected to the same level of stress with mannitol on Day 12 (Table 2).

Soluble carbohydrate composition. Glucose content of the cells was higher in all stress treatments compared with the control (Table 3). Cells subjected to both levels of glucose stress accumulated significantly higher levels of glucose than those subjected to the same level of mannitol stress. However, stress intensity had little effect on glucose content.

Although a general increase in the cellular fructose content was observed in all the stress treatments compared with the control (Table 3), significantly higher (74.0 nmol · cm^3 PCV^{-1}) fructose level was observed in −1.1 MPa glucose stress treatment compared with both control cells and the remaining stress treatments. Sucrose content in the cells of all stress treatments was significantly higher than that of the control (Table 3). The sucrose content of cells increased with the increase in the level of mannitol stress, but no such relationship was observed between the glucose stress levels and sucrose content. Glucose stress at −1.1 MPa (684 nmol · cm^3 PCV^{-1}) and mannitol stress at −1.6 MPa (678 mmol · cm^3 PCV^{-1}) induced significantly higher amounts of sucrose accumulation in the cells than the remaining treatments.

The inositol content of the cells was significantly higher in all stress treatments compared with that of the control (Table 3). Cells stressed with −1.1 MPa glucose had the highest amount of inositol (119.4 nmol · cm^3 PCV^{-1}), which was significantly higher than the remaining treatments. No significant difference in the inositol content of cells was observed between the two mannitol stress levels.

Mannitol-stressed cells were found to accumulate mannitol proportionally to the stress level (Table 3). As mannitol was found to accumulate in the cells, its metabolism was determined by feeding D-mannitol [U-^{14}C] on Day 12. Radioactivity was detected in the carbon dioxide evolved from the cells. Cells stressed with −1.1 MPa metabolized mannitol at the rate of 0.022 mmol manni-

TABLE 1

WATER AND OSMOTIC POTENTIALS OF OSMOTICALLY STRESSED AND CONTROL POPLAR CELLS AT DIFFERENT DAYS AFTER CULTURE INOCULATION[a]

	Water Potential (−MPa)				Osmotic Potential (−MPa)			
	Harvest Day							
Treatment	3	6	9	12	3	6	9	12
Control	0.67 (0)	0.66 (0.01)	0.57 (0)	0.45 (0.02)	0.95 (0.1)	0.89 (0.2)	0.87 (0.02)	0.6 (0)
A	1.24 (0.04)	1.16 (0.06)	1.22 (0.1)	1.09 (0.17)	1.69 (0.16)	1.4 (0.04)	1.4 (0.02)	1.49 (0.25)
B	1.69 (0.04)	1.74 (0.07)	1.8 (0.04)	1.96 (0.19)	2.11 (0.04)	2.09 (0.04)	2.13 (0.04)	2.14 (0.12)
C	1.15 (0.14)	1.09 (0.06)	0.94 (0.07)	0.78 (0.02)	1.58 (0.1)	1.41 (0.01)	1.27 (0.1)	1.15 (0.02)
D	1.55 (0.02)	1.71 (0.02)	1.69 (0.06)	1.76 (0.08)	2.09 (0.09)	2.16 (0.04)	2.00 (0)	1.91 (0.02)

[a] Control medium osmotic potential = −0.6 MPa. Means (n = 3) and SE are shown. LSD (P = 0.05) for mean comparison = 0.227 for water potentials and 0.27 for osmotic potentials. *A*, glucose −1.1 MPa; *B*, glucose −1.6 MPa; *C*, mannitol −1.1 MPa; *D*, mannitol −1.6 MPa. Water and osmotic potentials at harvest Day 0 measured in all cells −0.7 MPa and −1.17 MPa, respectively.

tol · h^{-1} (mg · cell dry weight)$^{-1}$ and those with −1.6 MPa at the rate of 0.116 mmol mannitol · h^{-1} (mg · cell dry weight)$^{-1}$. However, most of the mannitol was found to be present in the medium on the last harvest day, i.e. 212 mmol · ml^{-1} out of an initial level of 257 mmol · ml^{-1} in −1.1 MPa treatment (82%) and 459 mmol · ml^{-1} from an initial level of 498 mmol · ml^{-1} in −1.6 MPa treatment (92%).

Discussion

Accumulation of anthocyanin in the lag phase and the decrease in anthocyanin level subsequent to higher growth rate indicates an inverse relationship between growth and secondary metabolism in this culture. Such a relationship has been observed in most cell cultures (Sakuta and Komamine, 1987). Osmotic stress-induced inhibition of cell growth in this culture seems to be due to the inhibition of cell division (Fig. 2 *b*). The inhibition of cell division by osmotic stress in cell suspension culture has been previously reported (Do and Cormier, 1991). In the present study, osmotic stress resulted in an increase in the anthocyanin content of the cells (Figs. 2 *c* and 3 *a*, *b*, *c*). This anthocyanin accumulation was not a stress shock response because the culture continued to accumulate high anthocyanin levels on repeated subculturing in the low osmotic potential medium. An inverse relationship between growth and secondary metabolite accumulation has been demonstrated using inhibitors of cell division (Lindsey and Yeoman, 1985) and under osmotic stress conditions (Do and Cormier, 1990). This inverse relationship might be due to the diversion of metabolites from primary metabolism to secondary metabolism under growth-suppressing conditions (Fowler, 1986). In the present study, both levels of initial osmotic stress induced by glucose caused greater growth inhibition and higher anthocyanin content in the cells compared with the same level of mannitol stress. The difference in anthocyanin content of the cells between glucose and mannitol stress treatments became apparent only after the initiation of cell growth. Hence, the difference in the effect of glucose and mannitol stress on anthocyanin content of cells may be due to the difference in their effect on growth.

An increase in the anthocyanin content of a unit volume or weight of cells could occur due to an increase in the proportion of cells accumulating anthocyanin or due to an increase in the anthocyanin concentration in anthocyanin-producing cells. Determination of the percentage of pigmented cells (Fig. 3 *a*) and the anthocyanin content per pigmented PCV (Fig. 3 *b*) indicated that both are responsible for the higher anthocyanin content of a unit volume of cells brought about by osmotic stress. Anthocyanin per pigmented PCV is taken as an indicator of anthocyanin concentration in pigmented cells. The increase in the anthocyanin content per unit pigmented PCV by osmotic stress is not merely due to the observed decrease in the cell size brought about by osmotic stress conditions, but also to the actual increase in the anthocyanin content of the anthocyanin-producing cells as indicated by the increase in the anthocyanin per pigmented cell (Fig. 3 *c*). A comparison of the effects of glucose and mannitol stress indicated that an increase in the proportion of cells accumulating anthocyanin is largely responsible for the higher anthocyanin level in mannitol-stressed cells (Fig. 3 *a*), whereas an increase in the concentration of anthocyanin in the pigmented cells is responsible for the higher anthocyanin content of glucose-stressed cells (Fig. 3 *b*, *c*). Not withstanding the above, it is also possible that the osmotic stress is acting selectively in favor of the pigmented cells, at the expense of the non-pigmented ones. This aspect needs to be examined.

The decreased water potential of the cells (Table 1) seems to be responsible for the reduced growth and higher anthocyanin content of the cells subjected to osmotic stress. Water stress is known to decrease growth and increase secondary metabolite levels (Gershenzon, 1984). Differences in the water potentials and osmotic potentials of cells between the same initial osmotic stress levels induced by glucose and mannitol occurred only after the commencement of cell growth. An increase in the osmotic potential of the medium occurring due to higher growth and accompanied higher uptake and utilization of nutrients for cell growth, might explain the higher water potentials and osmotic potentials of the cells stressed with mannitol. This becomes evident by a comparison of the medium osmotic potential, cell water potential, and cell osmotic potential between −1.1 MPa glucose and mannitol stress treatments. Therefore, the difference in the cell water potentials and osmotic potentials between glucose and mannitol stress treatments probably occurred due to the difference in the effect of these osmotica on cell growth.

The osmotic potentials of the cells decreased in those treatments that decreased the cell water potentials. This indicates an increase

TABLE 2

SOLUBLE SUGAR CONTENT, FREE AMINO ACID CONTENT, AND SOLUBLE SUGAR-TO-AMINO ACID RATIOS IN POPLAR CELLS AFTER SUBJECTING CELL CULTURES TO OSMOTIC STRESS FOR UP TO 12 DAYS WITH DIFFERENT CONCENTRATIONS OF GLUCOSE AND MANNITOL[a]

	Soluble Sugars ($\mu g \cdot cm^3$ PCV^{-1})				
	Harvest Day				
Treatment	0	3	6	9	12
Control	656 (0)	732 (109)	1082 (24)	1206 (10)	798 (65)
A	656 (0)	905 (1)	1378 (158)	2591 (314)	4473 (208)
B	656 (0)	665 (3)	1413 (115)	2388 (52)	3347 (235)
C	656 (0)	928 (8)	1462 (78)	2138 (42)	2822 (228)
D	656 (0)	795 (52)	1530 (27)	2626 (196)	3669 (246)
	Amino Acids ($\mu g \cdot cm^3$ PCV^{-1})				
	Harvest Day				
Treatment	0	3	6	9	12
Control	668 (0)	473 (19)	503 (4)	779 (22)	900 (1)
A	668 (0)	475 (23)	490 (55)	1034 (138)	2075 (113)
B	668 (0)	278 (3)	558 (4)	724 (28)	1177 (120)
C	668 (0)	487 (17)	509 (26)	869 (58)	1649 (239)
D	668 (0)	387 (1)	564 (37)	825 (91)	1186 (68)
	Soluble Sugars: Amino Acids				
	Harvest Day				
Treatment	0	3	6	9	12
Control	0.98 (0)	1.53 (0.17)	2.15 (0.06)	1.55 (0.05)	0.88 (0.06)
A	0.98 (0)	1.99 (0.10)	2.81 (0.03)	2.52 (0.12)	2.17 (0.13)
B	0.98 (0)	2.45 (0.26)	2.53 (0.03)	3.30 (0.08)	2.86 (0.13)
C	0.98 (0)	1.91 (0.09)	2.88 (0.12)	2.48 (0.13)	1.74 (0.13)
D	0.98 (0)	2.06 (0.41)	2.74 (0.23)	2.74 (0.88)	3.09 (0.03)

[a] Means (n = 3) and standard errors are shown. *A*, glucose −1.1 MPa; *B*, glucose −1.6 MPa; *C*, mannitol −1.1 MPa; *D*, mannitol −1.6 MPa. LSD ($P \leq 0.05$) for mean comparison = 414 for soluble sugars and 223 for free amino acids.

in concentration of solutes in cells due to water loss or active solute accumulation. Accumulation of osmotic solutes prevents the loss of turgor (Borowitzka, 1981) but may also directly or indirectly affect secondary metabolism (Gershenzon, 1984). Sugars and amino acids are the important groups of solutes accumulated by plant cells during osmoregulation (Borowitzka, 1981; Zwiazek and Blake, 1990) and the levels of both solutes increased in osmotically stressed cells. The cellular content of glucose, fructose, sucrose, and inositol was higher in all stress treatments compared with the control on Day 12 (Table 3), when differences in the osmotic potential and anthocyanin content of the cells became most pronounced. Therefore, an increase in all of these individual sugars contributed to the increase in the soluble sugars, decrease in the osmotic potentials, and hence decreased growth and increased anthocyanin content of the stressed cells. However, among the stress treatments, sugar and anthocyanin levels could not be correlated.

It has been reported that increased supply of sugars and decreased supply of nitrogen through the medium caused a reduction in cell growth and an increase in anthocyanin levels in grape cell suspension culture (Do and Cormier, 1991). Therefore, in addition to their individual quantities, the ratio of soluble sugars to free amino acids might also be important in influencing growth and anthocyanin accumulation. An increase in this ratio may create an imbalance in the solute composition of the cells resulting in an inhibition of cell growth and increase of anthocyanin production. In the present study, the ratio of soluble sugars to free amino acids in cells increased proportionally to stress level. However an exact comparison of the total free sugars between glucose and mannitol stress treatments using colorimetric methods may be obscured due to different reaction intensities of the individual sugars (Ashwell, 1957). Subsequent analysis of soluble carbohydrate composition of the cells showed that the sucrose content of cells stressed with −1.6 MPa mannitol was significantly higher than that of −1.6 MPa glucose stress treatment on Day 12. Thus, the reason for the higher ratio of soluble sugars to amino acids found in −1.6 MPa mannitol-stressed cells compared with those stressed by the same level of glucose could be because of the higher values for sucrose than for monosaccharides obtained by the anthrone method (Ashwell, 1957).

The experiment with [U-^{14}C]mannitol showed that cells in the present culture were able to absorb and metabolize some of the mannitol present in the medium. However, the presence of most of the mannitol in the medium on Day 12 indicated that mannitol was not taken up by these cells in substantial amounts. Therefore, comparison of the osmotic effects of glucose and mannitol in this culture is in fact a comparison between an osmoticum that is readily taken

TABLE 3

COMPOSITION OF SOLUBLE CARBOHYDRATES IN OSMOTICALLY STRESSED AND CONTROL POPLAR CELLS 12 DAYS AFTER CULTURE INOCULATION[a]

	Soluble Carbohydrate ($nmol \cdot cm^3$ PCV^{-1})				
Treatment	Glucose	Fructose	Sucrose	Inositol	Mannitol
Control	8.7 (2.4)	18.5 (6.4)	24.6 (16.0)	19.5 (3.4)	0
A	110.6 (28.2)	74.8 (11.1)	684.2 (35.8)	119.4 (4.4)	0
B	104.9 (5.4)	30.3 (2.3)	540.8 (65.4)	53.7 (7.9)	0
C	47.0 (10.5)	35.4 (0.6)	299.5 (27.2)	89.6 (10.9)	424.7 (56.7)
D	58.6 (8.2)	30.3 (2.5)	677.7 (50.0)	94.8 (8.9)	656.8 (103.5)
LSD	44.8	18.7	134.1	23.4	166.4

[a] Means of three replicates are shown with SE in parentheses. *A*, glucose −1.1 MPa; *B*, glucose −1.6 MPa; *C*, mannitol −1.1 MPa; *D*, mannitol −1.6 MPa. Least significant difference (LSD, $P \leq 0.05$) values for mean comparison are presented.

up and metabolized (glucose) and an osmoticum that is slowly taken up (mannitol) (Thompson et al., 1986) and metabolized. However, because the maximum anthocyanin accumulation in this culture took place in the lag phase, carbon source is not likely to be a limiting factor at that time. Therefore, the different degrees of metabolism of glucose and mannitol may not have influenced anthocyanin production in this culture. In a separate experiment we used polyethylene glycol 3350 to induce osmotic stress in cell culture and obtained a similar accumulation of anthocyanin to that in glucose- and mannitol-treated cells (data not shown), indicating that osmotic stress is a primary factor responsible for this response. Mannitol is considered to have little effect on enzymes and hence is considered as a compatible solute (Borowitzka, 1981). Therefore, accumulation of mannitol in the mannitol-stressed cells might have resulted in higher cell growth and consequently in lower anthocyanin levels compared with those stressed to the same level by glucose.

Significant differences in cell water potential, osmotic potential, soluble sugar to amino acid ratio, and anthocyanin content between glucose- and mannitol-stress treatments occurred subsequent to growth initiation. Therefore, solute composition just before the commencement of growth might play an important role in influencing the growth rate of glucose- and mannitol-stressed cells. The cells stressed by -1.1 MPa mannitol were found to accumulate mannitol (75 nmol $\cdot$ cm^3 PCV^{-1}) on Day 6, just before the commencement of growth. This observation again indicated that the presence of mannitol, a compatible solute, in mannitol-stressed cells might be responsible for the difference in growth and hence anthocyanin levels observed between the glucose- and mannitol-stress treatments.

We can conclude that both the extent of osmotic stress and the type of solute used to induced osmotic stress seem to influence anthocyanin levels in this culture through their influence on cell growth. Osmotically induced growth inhibition could cause an inhibition of primary metabolism and the consequent diversion of metabolites for anthocyanin biosynthesis. The solutes accumulated due to growth inhibition might, in turn, influence anthocyanin production by inducing the activity of the regulatory enzymes for anthocyanin biosynthesis. Hence, an understanding of the cellular composition of other groups of solutes that are known to increase due to growth inhibition under osmotic stress condition, such as amino acids and organic acids, and the activity of the regulatory enzymes of anthocyanin biosynthesis might be helpful in further elucidating the mechanism of osmotically induced anthocyanin production.

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