

Effect of sorbitol induced osmotic stress on the changes of carbohydrate and free amino acid pools in sweet potato cell suspension cultures

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Abstract. The effects of osmotic stress induced by 0.6 M sorbitol on the cell growth and on the quantitative and qualitative changes in carbohydrates and free amino acids in suspended cells of sweet potato (*Ipomoea batatas*) were analyzed. Cells transferred into medium without (normal treatment) or with (stress-shocked treatment) 0.6 M sorbitol added, and cells consecutively subcultured under high stress conditions (stress-adapted treatment) were compared. Stress-shocked cells showed cell growth retardation and the induction of plasmolysis. Stress-adapted cells had a shorter lag phase in growth than the stress-shocked, and showed a normal morphology, albeit the size appeared slightly smaller than normal cells. Under the stress-shocked condition, the size of the amino acid pool ($\mu\text{mole/g}$ fresh weight) increased fourfold relative to the control and stress-adapted cells. The levels of alanine and glutamic acid and its derivatives were especially high, indicating that the changes in the intensity of glycolysis have influenced the amino acid pool. Although the proline level showed a fivefold increase when stress-shocked, proline made up only about 1.5% of total amino acids, and thus did not seem to play an osmotic regulatory function. Among the carbohydrates, sucrose content was high in both stress-shocked and stress-adapted cells. Starch accumulated heavily in stress-shocked cells, but not in normal or stress-adapted cells, although the latter maintained a higher background level of starch. It is tempting to speculate that sucrose serves as a compatible solute, and starch synthesis from sucrose plays a pivotal role in moderating the hyperosmotic condition. The accumulated starch contained less amylose than the ordinary tuberous root starch, indicating that the pathway of starch synthesis was somewhat altered in the stress-shocked cells.

Keywords: Compatible solute; Free amino acid; *Ipomoea batatas*; Osmotic stress; Starch; Sucrose; Suspended cells.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GABA, γ -aminobutyric acid; MS, Murashige-Skoog.

Introduction

Plants resort to many adaptive strategies in response to abiotic environmental stresses such as high salt, dehydration, cold, heat, and excessive osmotic pressure. These adaptive mechanisms include changes in morphological and developmental patterns as well as physiological and biochemical processes (McCue and Hanson, 1990). Among them, the accumulation of compatible solutes according to the metabolic responses has drawn much attention. Some stress-responsive genes encoding proteins for compatible solute synthesis have been cloned and expressed in transgenic plants (Tarczynski et al., 1993; Bartels and Nelson, 1994; Kavi Kishor et al., 1995). The compatible solutes may be classified into two categories:

one is nitrogen-containing compounds such as proline and other amino acids, quaternary ammonium compounds and polyamines, and the other is hydroxy compounds, such as sucrose, polyhydric alcohols, and oligosaccharides (McCue and Hanson, 1990). The species of accumulated solutes varies with the variation in adverse conditions and plant species, or even plant varieties.

In general, a plant cell suspension culture is considered a relatively homogeneous population of cells. Much research has used cultured cells as a model system to study the cellular responses under various abiotic stress, even to distinguish the difference between the short-term response and long-term adaptation involving physiological and biochemical changes (Fallon and Phillips, 1989; Leone et al., 1994).

Sorbitol is an alditol found in higher plants. It is the major photoassimilate in most species of *Rosaceae* (Moing et al., 1992) and the main low molecular weight saccharide

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found in *Plantago* after exposure to salinity (Ahmad et al., 1979). However, sorbitol has been considered a non-metabolite, because it is metabolically more inert than other saccharides (Lambers et al., 1981).

In order to look into osmotic stress induced biochemical changes and to elucidate adaptive mechanisms at the cellular level, we used a high concentration of sorbitol (0.6 M) as osmoticum to investigate the status of carbohydrate and amino acid pools in sweet potato cells grown under normal and high stress media (stress-shocked), and in cells consecutively cultured on high concentrations of sorbitol (stress-adapted).

Materials and Methods

Plant and Cell Culture

The suspension cells were derived from a callus tissue which was induced from the tuberous root of sweet potato, *Ipomoea batatas* cv. Tainong 57 (Wang et al., 1993). The cells were maintained in Erlenmeyer flasks containing MS medium (Murashige and Skoog, 1962) supplemented with 9 μ M (2 ppm) 2,4-D, 0.9 μ M (0.2 ppm) kinetin and 3% (w/v) sucrose at pH 5.6 before autoclaving. Cells were cultured on a rotary shaker at 120 rpm in the dark at 25°C, and were subcultured every 7 days. Cells were established and subcultured over at least eight transfers.

Several papers report a reciprocal relationship between the levels of nitrogen and the starch content in algae and higher plants (Miyachi and Miyachi, 1985; Rufty et al., 1988; Huppe and Turpin, 1994). Hence, in order to study the effect of osmotic stress on carbon's assimilation into starch, we initially changed the basal medium from MS salts with 1.9 g/L of NH_4NO_3 to Gamborg's B5 salts (Gamborg et al., 1968), in which no NH_4NO_3 was present. However, the suspended cells aggregated, and the growth was poor under no NH_4NO_3 conditions. Thus, the B5 salts were modified by adding NH_4NO_3 to 0.4 g/L and decreasing KNO_3 from 2.5 g/L to 2.0 g/L. Unless indicated otherwise, the experiments for investigating osmotic effect used modified B5 salts containing 10 μ M (2.2 ppm) 2,4-D and 3% (w/v) sucrose with (high stress) or without (normal) 0.6 M sorbitol added.

In this study, three types of cells were compared. The first type were cells maintained in MS medium and transferred into the modified Gamborg's medium without sorbitol. These were referred to as normal cells. The second type were cells treated as above but transferred into the 0.6 M sorbitol containing medium. They were designated as stress-shocked. The third type was continuously cultured in the modified Gamborg's medium containing 0.6 M sorbitol, with a transfer every 3 weeks for a total of at least 15 transfers. These cells were designated as stress-adapted.

At the end of the experiments, cells were rapidly washed under an aspirator-suction with respective media from which sucrose was omitted. The growth rate of suspended cells was monitored by measuring the fresh weight and packed cell volume.

Chemical Analysis

For soluble sugar determination, the washed cell was homogenized and extracted with hot 80% (v/v) ethanol. The extract was centrifuged. The supernatant was evaporated in an N_2 gas stream, then dissolved in deionized water and filtered through Millipore Millex-GX nylon membrane. Sugars were separated on a CarboPac PA1 column (4 \times 250 mm, Dionex) using 70 mM NaOH as eluant and quantified in a high pH anion exchange chromatograph with a pulsed amperometric detector (Dionex). The sediment was analyzed for starch as described by Wang et al. (1993). The amylose content of starch was determined by dual-wavelength spectrophotometry according to the method described by Hovenkamp-Hermelink et al. (1988). Briefly, a sample of 100~150 mg washed cells or 10~30 mg fresh root crushed in a tube with a glass rod was mixed with 0.5 mL of 45% HClO_4 and shaken. After 4 min, 8 ml of H_2O were added. The supernatant was mixed with a diluted (1:2, v/v) Lugol solution, and OD values at 618 nm and 550 nm were immediately measured. Amino acid was extracted by 80% ethanol (5 mL per 100 mg washed cells) following the method of Jackson and Seppelt (1995). The extract was filtered through a Millipore Ultrafree-MC polysulfone membrane (cutoff Mr, 10 k) to remove proteins. Filtrates were lyophilized and kept as sample stocks. Amino acid analysis was done by a ninhydrin system using a Beckman 6300 amino acid analyzer with a single ion-exchange column.

Microscopic Observation

Starch deposition in the suspension cell was observed microscopically after iodine staining.

Osmolality Measurement

The osmotic pressure of medium or supernatant was measured using a cryoscopic osmometer from Roebbling (Berlin, Germany).

Results and Discussion

Osmotic Stress Effects on Cell Growth and Morphology

The growth of stress-shocked cells was severely retarded (Figure 1A and B). In contrast, the growth of normal cells was greatly enhanced 3 days after the transfer into normal medium when the osmolality of medium decreased (Figure 1C).

In preliminary tests, we found the cells grew slightly under high osmotic stress when cultured for 3~4 weeks, so a stress-adapted cell line was established. As shown in Figure 1A and B, adapted cells characteristically showed a shorter lag phase, compared with the maintenance cells abruptly exposed to high osmotic conditions, and a significantly increased growth rate 7 days after being transferred into a fresh stressed medium.

Based on the sugar analysis, sorbitol concentrations in the culture media of shocked or adapted cells remained

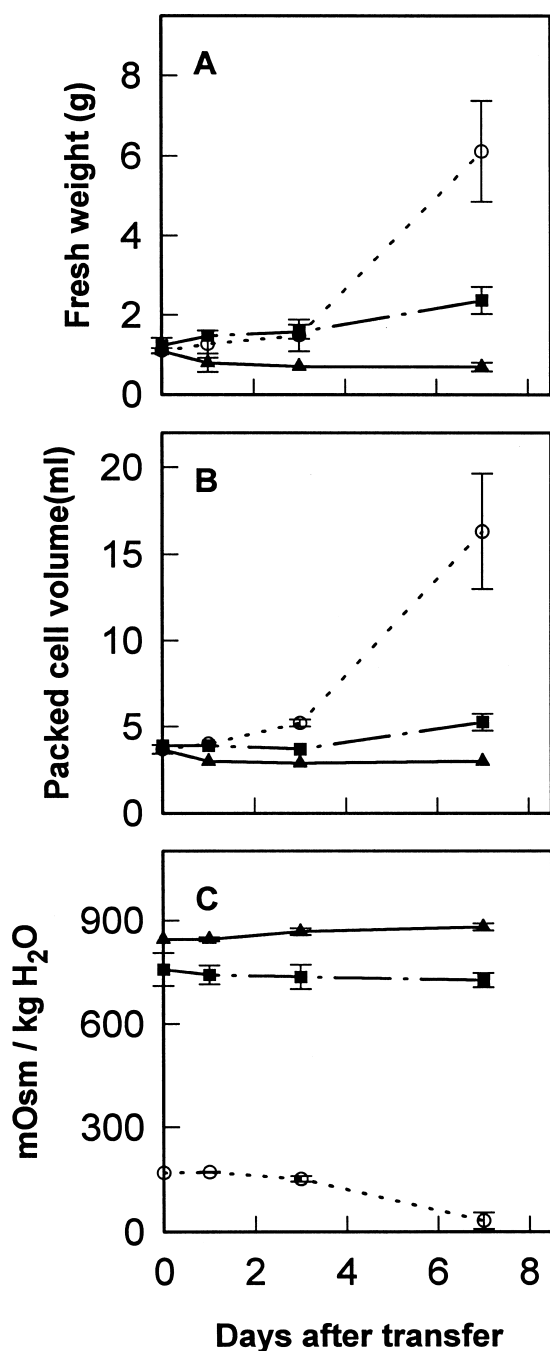


Figure 1. Changes in fresh weight (A), packed cell volume (B) of suspended cells and the osmolality (C) of supernatant from normal (○), stress-shocked (▲) and stress-adapted (■) cells. Each point is the average of at least two independent samples.

unchanged, indicating that sorbitol was not metabolizable by suspension-cultured sweet potato cells (data not shown). We may thus say that sorbitol served as the osmoticum only.

In addition to retarding cell growth, stress-shock caused plasmolysis, the separation of plasmalemma from the cell wall (Figure 2B). In contrast, the stress-adapted cell had a normal morphology although they were slightly smaller than the normal cells (Figure 2A and C). The reduction of

cell size in cultured suspension cells adapted to salinity was also found in *Citrus sinensis* (Ben-Hayyim and Kochba, 1983) and tobacco (Binzel et al., 1985) cells. Binzel et al. (1985) suggested that adaptation involved considerable osmotic adjustment with increased Turgor but reduced cell expansion.

Osmotic Stress Effects on Amino Acid Pool

The concentrations of amino acids from three types of sample three days after being transferred into their respective media were compared in Table 1. In stress-shocked cells, the size of the amino acid pool was three to four times normal. Similar results were reported for salinity-, drought- or osmotic-stressed plants and tissue cultures (Galiba et al., 1989; Fougere et al., 1991; Good and Zaplachinski, 1994; Cano et al., 1996; Gzik, 1996). However, the amino acid contents of stress-adapted and normal cells showed no marked differences. We may say that the stress-shock effect on the amino acid metabolism will return to normal after the cell adapts to the stress.

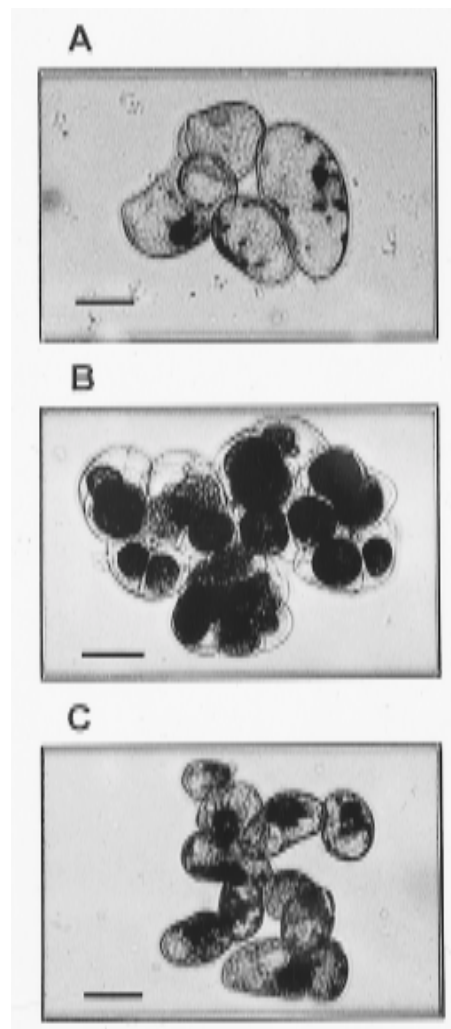


Figure 2. Morphology of normal (A), stress-shocked (B) and stress-adapted (C) cells after 7 days subculture in respective media and stained with an iodine reagent. Bar is 40 μm.

Alanine is the most abundant amino acid, reaching 24%, 35% and 22% of the total amino acid in normal, shocked, and adapted cells, respectively (Table 1). The stress-shock resulted in a fivefold increase in alanine content on the third day. When the cells were cultured in the maintenance medium, alanine occupied 60% of the amino acid pool or 42 $\mu\text{mole/g}$ fresh weight on the seventh day after the medium change. The alanine level decreased when the cells were transferred from N-rich MS-medium into N-poor modified Gamborg medium, regardless of whether the stress agent sorbitol was added or not. However, alanine decreased much more under normal conditions, and the osmotic stress was effective in preventing the turning over of alanine. In maize callus (Santos et al., 1996) and some species of seagrass (Pulich, 1986), salinity also elevated the level of alanine. Whether alanine itself, or the metabolic pathway leading to the change of its level, acts as a regulatory mechanism of osmotic and other forms of physiological stress is an interesting question.

Glutamine and glutamate are also major amino acids in sweet potato cells. When the ratios of glutamate to glutamine among the three types of cell were compared, the stress-shocked cells had the lowest value of 0.34 while the normal was 0.50 and the adapted was 0.81. The same trend was found for sugar beet leaf discs exposed to polyethylene glycol (PEG) (Gzik, 1996). In *Vigna radiata* calli (Gulati and Jaiwal, 1996) and *Vicia faba* (Cordovilla et al., 1996), salinity stress inhibited glutamate synthase, which catalyzed the reaction of glutamine + α -ketoglutarate \rightarrow 2

glutamate. Therefore, it is tempting to conclude that the decrease of glutamate synthase activity may be one of the reasons for the lowering of the ratio of glutamate to glutamine in sweet potato cells under a high osmotic condition.

γ -Aminobutyric acid (GABA) and serine are also abundant in sweet potato cells, and osmotic shock caused 2.8- and 4.2-fold increases in them, respectively. However, the rates of increase mirrored those of the total pool size, so their relative levels in the pool remained about the same. GABA is derived from glutamate, and serine shares the same synthetic route with alanine. All of the above mentioned amino acids are related to the initial steps of amino acid synthesis from the two important α -keto acids, pyruvate and α -ketoglutarate, derived from glycolysis and the tricarboxylic acid (TCA) cycle, respectively. We thus propose that one short term effect of osmotic stress is to exert a great disturbance on the ammonia assimilation pathways in sweet potato cells.

Among amino acids, the accumulation of proline, another glutamate family amino acid, is frequently reported in many plants or tissues in response to a variety of abiotic stresses (Hare and Cress, 1997). In the maize primary root, for example, the proline level increases as much as a hundred fold under a low water potential (Voetberg and Sharp, 1991). However, the precise role of proline accumulation is still elusive. Whether it is to act as an osmoregulator (Delauney and Verma, 1993), an osmo-protector (Csonka, 1989), or a regulator of the redox potential of cells (Bellinger and Larher, 1987) has not been decided.

The levels of proline were low and comparable in normal and stress-adapted cells, but the stress-shocked cells showed a fivefold increase (Table 1). However, proline is only about 1.5% of the total amino acid content. This amount is not high enough to convincingly represent a physiological function.

Usually the magnitude of proline accumulation is relatively dependent on the levels of carbohydrates (Stewart, 1978; Larher et al., 1993; Hare and Cress, 1997). Larher et al. (1993) mentioned that sucrose was a positive effector of proline accumulation. As we will discuss later, the level of sucrose was greatly enhanced and yet the level of proline remained low. The significance of proline in the stress-shocked sweet potato cells cannot be over-emphasized.

Osmotic Stress Effects on Carbohydrate Levels

The accumulation of sugars in response to applied stress conditions is also quite well documented (Gorham et al., 1981; Yancey et al., 1982; Zrenner and Stitt, 1991; Kameli and Losel, 1993). Figure 3 shows that the total sugar content (the sum of glucose, fructose, and sucrose) under different stress conditions at day 7 was not significantly different. Although stress-shocked cells slightly decreased the levels of glucose and fructose, the concentration of sucrose was sharply enhanced. Relative to normal cells, sucrose level increased fourfold in shocked cells and threefold in adapted cells. In normal cells, the sucrose content accounted for 23% of the total sugar pool, pro-

Table 1. Free amino acids composition of normal, stress-shocked and stress-adapted sweet potato cells cultured under respective media for 3 days. All values are in $\mu\text{mole/g}$ fresh weight. Each value is the average of 2 independent samples.

Amino acid	Normal	Stress-shocked	Stress-adapted
Asp	0.334	0.812	0.308
Thr	0.238	0.600	0.104
Ser	0.728	3.072	0.846
Asn	0.188	0.430	0.359
Glu	0.803	1.665	1.534
Gln	1.616	4.814	1.902
Pro	0.077	0.404	0.079
Gly	0.217	0.492	0.056
Ala	1.925	9.432	1.758
Val	0.272	0.950	0.122
Met	0.014	0.022	0.027
Ile	0.085	0.149	0.033
Leu	0.119	0.290	0.038
Tyr	0.030	0.032	0.009
Phe	0.051	0.062	0.054
GABA	0.881	2.480	0.428
β -Ala	0.012	0.073	0.010
Trp	0.003	0.021	0.002
Orn	0.041	0.042	0.004
Lys	0.072	0.077	0.027
His	0.176	0.307	0.075
Arg	0.102	0.278	0.024
Total	7.984	26.504	7.799

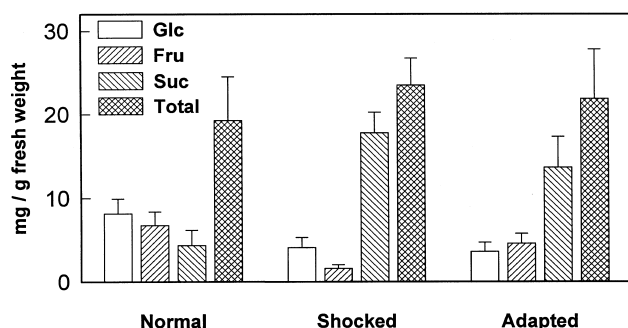


Figure 3. Sugar content of normal, stress-shocked and stress-adapted cells after 7 days subculture in respective media. Total sugar was the sum of glucose, fructose, and sucrose. All values are the average of at least two independent samples.

foundly lower than the 76% for shocked and 62% for adapted cells. Hence, sucrose might be considered as a compatible solute for sweet potato cells exposed to osmotic stress.

Besides increasing the sucrose content, stress shock also induced a large starch accumulation and maintained the high starch content for a long period of time (Figure 4). Conversely, under normal conditions, the starch level rose following the transfer into a fresh medium, and then declined rapidly (Figure 4). On the other hand, stress-adapted cells, 7 days after being transferred into the fresh medium, had a lower level of starch (13.5 mg/g fresh weight) than the shocked (27.2 mg/g fresh weight) but a higher level of starch (4.3 mg/g fresh weight) than the normal cells.

All these data taken together indicated that sucrose and starch have a good corresponding relationship as

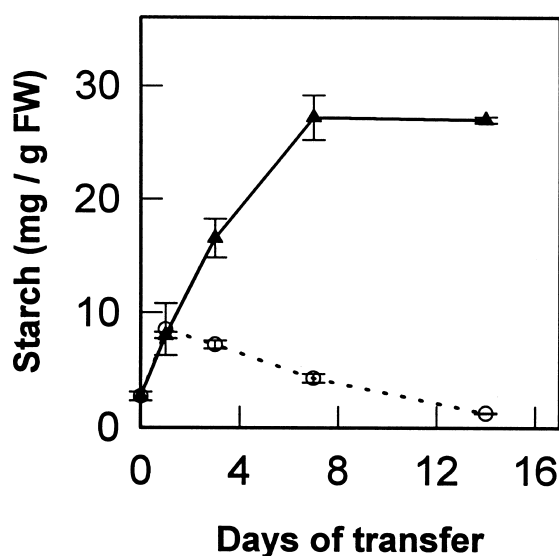


Figure 4. Changes in starch content of suspension cells under stress-shocked (▲) and normal (○) conditions. Each point is the average of at least two independent samples.

Table 2. Amylose fraction in stress-shocked cells and fresh tubers of sweet potato cv. Tainong 57. Values are the means of 8 samples \pm SE.

	618 nm/550 nm	Amylose % ^b
Tuber	1.064 \pm 0.053	22.1
Cells ^a	0.907 \pm 0.019	10.7

^aCells were cultured in high stress medium for 7 days.

^bAmylose fraction = (3.5-5.1 R) / (10.4 R-19.9), R is the ratio of the absorbancies at 618 and 550 nm.

source and sink substances, respectively, in higher plants, and the observed phenomena may be explained as follows. A much enhanced accumulation of starch in stress-shocked cells may be seen as the consequence of a sudden surge in accumulation of the compatible solute sucrose, a large excess of which is directed toward the synthesis of sink substance starch. On the other hand, the elevation in starch accumulation was not so significant in stress-adapted cells although they also had a significant increase in sucrose by being transferred into a fresh stressed medium. This is probably because the adapted cells are so well acclimated to the osmotic stress condition and reduce the partitioning of sucrose toward starch. Osmotic shocks exerted by high salinity (120 mM NaCl) or sucrose (388 mM) levels also induced starch accumulation in sweet potato cells (data not shown). The formation of starch was also found in rice callus subjected to 0.6 M sorbitol or mannitol (Liu and Lai, 1991). Therefore, it is reasonable to assume that, being an effective carbon sink in response to the elevated availability of a carbon source, or sucrose, starch may also play an important role in moderating the osmotic shock-induced accumulation of sucrose in cells to a physiologically appropriate level.

Additionally, the starch accumulated in the osmotic-shocked cells had less amylose than usual, as indicated by the lower ratio of light absorbance values at 618 nm and 550 nm of the iodine complex (Table 2). The amylose content as a percentage of total starch was calculated (Hovenkamp-Hermelink et al., 1988), and the value was only one-half that of the percentage in ordinary sweet potato starch.

Under stress-shocked conditions, we further postulate that the deposition of starch granules together with induced plasmolysis will reduce the volume of cytoplasm. Hence, accumulation of only a small amount of sucrose may be adequate to counter the osmotic stress imposed upon the cells. Why the accumulated starch had a higher branched structure, and how the metabolic flux of carbon sources was altered under the stress conditions, which should involve modulation of many enzyme activities in carbohydrate metabolic pathways, remains to be investigated.

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山梨糖醇誘導的滲透逆境對甘藷懸浮培養細胞碳水化合物與自由態胺酸庫變化的影響

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本研究探討高濃度 (0.6 M) 山梨糖醇引起的滲透逆境，對於甘藷 (台農 57 號) 懸浮培養細胞的生長以及碳水化合物和自由態胺酸在定性與定量方面的影響。比較細胞分別移入培養基不含 (正常處理) 與含 (逆境衝擊處理) 山梨糖醇，以及細胞長期培養在高滲透逆境下 (逆境適應處理)。逆境衝擊阻礙細胞生長並且造成原生質分離。相較於逆境衝擊的細胞，適應逆境的細胞具有較短的生長遲滯期和正常型態，雖然稍小於正常處理的細胞。在逆境衝擊下，細胞內胺酸庫增加 4 倍，而以丙胺酸、麩胺酸及其衍生物之增加為顯著，顯示糖解代謝之改變對胺酸之合成發生影響。雖然脯胺酸的含量亦增加 5 倍，由於僅佔總胺酸的 1.5%，無法說明具有顯著的生理意義。在碳水化合物方面，高含量的蔗糖存在於逆境衝擊與逆境適應的細胞；而大量澱粉累積僅發生於受逆境衝擊的細胞，未表現於正常處理與適應逆境的細胞，雖然後者亦有稍高的澱粉含量。因此，合理推測蔗糖當作可親和性溶質，而且蔗糖轉換成澱粉的機轉在調節高滲透環境中扮演關鍵角色。由滲透逆境所累積澱粉的直鏈澱粉含量明顯低於來自新鮮甘藷塊根，表示遭受逆境衝擊的細胞在合成澱粉的途徑具有些許改變。

關鍵詞：可親和性溶質；自由態胺酸；甘藷；滲透逆境；澱粉；蔗糖；懸浮細胞。