ACTA
PHYSIOLOGIAE
PLANTARUM
Vol. 24. No. 4. 2002: 441-446

Relationship between sucrose accumulation and activities of sucrose-phosphatase, sucrose synthase, neutral invertase and soluble acid invertase in micropropagated sugarcane plants

Federico A. Gutiérrez-Miceli *, *Martha A. Rodríguez-Mendiola* **, *Neftalí Ochoa-Alejo* †, *Ricardo Méndez-Salas* ††, *Luc Dendooven* ‡ *and Carlos Arias-Castro***

Departamento de Biotecnología y Bioingeniería, CINVESTAV-IPN, Av. IPN 2508, CP 07360, México DF, México
* Laboratory of Vegetal Biotechnology, Instituto Tecnológico de Tuxtla-Gutierrez, Tuxtla-Gutierrez, México
** Ciatej, Av. Normalistas No 800, Colinas de la Normal, CP 44270, Guadalajara, Jalisco, México
† Unidad Irapuato del CINVESTAV, Km 9.6 Carr Irapuato-León, AP 629, CP 36500, México
†† Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Campo Experimental Zacatepec, Zacatepec, Morelos, México
‡ Departamento de Biotecnología y Bioingeniería, Cinvestav, Av IPN 2508, CP 07000, México DF

Key words: neutral invertase, soluble acid invertase, sucrose-phosphate synthase, sucrose synthase, sugarcane

Abstract

The activities of sucrose-phosphate synthase (SPS), sucrose synthase (SUSY), neutral invertase (NI) and soluble acid invertase (SAI) regulates sucrose activity in sugarcane were studied. Micropropagated sugarcane plants were obtained from callus cultures of four Mexican commercially available sugarcane varieties characterized by differences in sugar production, and activities of SPS, SUSY, NI, SAI and concentrations of sucrose were monitored in the sugarcane stem. The results indicated that sucrose accumulation was positively and significantly related to an increase in activity of SPS and SUSY and negatively to a reduction in activity of SAI and NI ($P<0.05$). SPS explained most of the variations found for sucrose accumulation and least for NI. The relationship between activity of SPS, SUSY, NI and SAI in sugarcane stem was similar in each variety.

List of abbreviations: NI neutral invertase, SAI soluble acid invertase, SPS sucrose-phosphate synthase, SUSY sucrose synthase

Introduction

Modern sugarcane cultivars are multispecies hybrids, primarily of *Saccharum officinarum* L., *Saccharum spontaneum* L., and *Saccharum robustum* Brandes et Jeswiet exGrassl. The parenchyma cells of sugarcane stem accumulate sucrose up to 20 % of their fresh weight or 60 % of dry weight in mature internodes (Bowen 1972). Stems of high sucrose accumulating clones are generally high in moisture and low in fibre (Bull and Glasziou 1963). Such stems have a thick girth and high fresh weight. Stems of low sucrose storing clones are generally thin and fibrous and have a low fresh weight (Moore and Maretzki 1996). Part of the dif-

ference in sucrose storage among genotypes might be based on morphological characteristics.

Sucrose in the stem can be catabolized by either sucrose synthase (SUSY, EC 2.4.1.13) or by one of three invertases: a soluble acid invertase (SAI, EC 3.2.1.26) high in apoplast and vacuoles of young internodes, but virtually absent in the mature tissues; an acid invertase (AI, EC 3.2.1.26) bound to the cell wall in tissues of all ages; and a neutral invertase (NI, EC 3.2.1.26), low in the cytoplasm of young tissues and high in mature tissues (Moore and Maretzki 1996). After entering the metabolic compartment of the parenchyma cells, the hexoses may be metabolized or resynthesized into sucrose by sucrose-phosphate synthase (SPS, EC 2.3.1.14) or sucrose phosphatase (SPase). SUSY may also be involved in sucrose synthesis, but the equilibrium is usually in the direction of degradation (Goldner *et al.* 1991). Studies to determine sucrose accumulation in the sugarcane are complicated and still a lot has to be unravelled about the biochemistry of sucrose accumulation (Grof and Campbell 2001). Additionally, plants are used of different ages, exposed to different environments, and from different genotypes which also affects sucrose accumulation (Moore 1995).

Micropropagation gives large amounts of plants with less variation in a shorter time compared to the traditional vegetative method. It is more and more frequently used commercially and sometimes sugarcane plants thus obtained show larger sucrose accumulation than the parent plant. Sucrose accumulation and activity of SPS were investigated in leaves of sugarcane (Grof *et al.* 1998) and in stem using vegetative generated plants (*e.g.* Zhu *et al.* 1997), but few reports exist about activities of SPS, SUSY, NI and SAI in micropropagated sugarcane plants. We, report here on sucrose accumulation and activities of SPS, SUSY, NI and SAI in micropropagated sugarcane of four genotypes from Mexico.

Materials and Methods

Plant material

Four sugarcane cultivars differing in sugar accumulation and sugar yield were obtained from the experimental field "Zacatepec" of Morelos of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), México. Rb 765418 and Mex 57-473 are characterized by a large sugar production, Mex 69-290 by a medium production and Zmex 55-32 by a low production. Callus cultures were developed from stalk apices of the four field-grown cultivars. Shoot tips of 15 cm long were disinfected in 95 % (v/v) ethanol for 30 s and 3 % (w/v) calcium hypochlorite for 30 min, and washed 3-times with sterile distilled water. The outer leaf sheaths were removed to about the tenth node, and the tender ones until only the innermost leaf sheaths remained. The two millimetres transverse sections were cut from the apex and placed on 30 cm^3 of MS semi-solid medium (Murashige and Skoog 1962). Four explants were placed in each 150-cm^3 bottle and incubated at 26-28 °C under cool white fluorescent light (45.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with 8/16 h light/darkness cycles.

The calli were cultivated on MS medium with 3 % (w/v) sucrose, 10 $g \cdot dm^{-3}$ activated charcoal, 1 $mg \cdot dm^{-3}$ arginine, 0.3 $g \cdot dm^{-3}$ yeast extract, 3 $mg \cdot dm^{-3}$ 2,4-D and 1.8 $g \cdot dm^{-3}$ Gelrite. The pH was adjusted to 5.8 prior to autoclaving at 128 kPa for 20 min. Calli with green meristematic regions were cultivated on the same medium, but without 2,4-D, to regenerate plants. Shoots developed after 30 days were separated and placed in 150-cm^3 bottles containing 30 cm^3 of the same medium. The plantlets were transferred to disinfected soil in unicel pots and covered with plastic cover. The plants were hardened over a 14-day period by gradually opening the plastic cover, after which the plants were maintained under greenhouse conditions. After 30 days, the regenerated plants were planted in the experimental field. The plants were grown using standard practices for water and fertilizer applications. After 3 months (September) and than until the 10th month (March) (Fig. 1), three stems were excised at the soil surface level monthly, the leaves were removed, and the internodes were numbered sequentially down the stalk according to their stage of development. The first internode taken from the top of the plant with a fully expanded leaf was designated internode 1, with lower internodes given sequentially increasing numbers (Clements and Ghotb 1968). The

stems were cut into individual internodes and the three internodes that made up the middle of the stem were selected. The internode ring, consisting of a thick epidermis and sclerified vascular bundles, was removed and the remaining internode was weighed and cut into quarters lengthwise. Each quarter was chopped into small pieces and frozen in liquid nitrogen before being stored in a freezer until used for sugar and enzyme assays.

Sucrose extraction and determination

Sucrose concentrations were measured on sub-samples of the same internode that were used for enzyme activity assays. Frozen, chopped pieces from each internode were divided into two portions. The sucrose was soxhlet-extracted with 100 cm^3 98 % ethanol for 12 h which gave a recuperation of 95-98 % (data not shown). The volume of the alcoholic extract was reduced in a 461 rotavapor (Buchi, USA) at 70 °C under vacuum. The extracts were dried in a vortex evaporator (Labconco, USA) at 40 rpm and 70 °C, and re-dissolved in 1 cm^3 acetonitrile:water (80:20) solution. Aliquots of 20 µl were analyzed for sucrose on an amino-silica column with a Varian HPLC (USA) fitted with a R1-4 refraction index detector and a 4400 integrator. Acetonitrile:water (80:20) was used as the mobile phase at a flow rate of 1.0 $cm^3 \cdot min^{-1}$. Sucrose concentration of the whole-stalk was the sum of concentration for internode 2 to 11. The data presented are the mean values from at least three independent extractions.

Enzyme extraction

Tissue frozen in liquid nitrogen was weighed and ground to fine powder in a chilled mortar with quartz sand (Sigma) to facilitate cell disruption. Extraction buffer containing 50 mM Hepes-NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM DTT, 2 mM benzamidine, 2 mM N-aminocapronate, and 10 mM diethyldithiocarbamate was added (Goldner *et al.* 1991). The ratio fresh/weight extraction buffer depended on the internode age: 0.2 to 0.3 g fresh weight·cm^{-3} for internodes 1 to 4, and 0.4 g fresh weight·cm^{-3} for internode 5 and older. The tissues were sonicated for 30 min in the extraction buffer to recover cell wall-bound invertase. All the extracts were desalted by dialysis using spectrapore membranes (Thomas Scientific, USA) and partially purified on a micro-scale w-amino-hexyl-agarose column, as described by Dancer *et al.* (1990). Recovery of the soluble enzymes (SUSY, SPS, and invertases) was approximately 75 % (Zhu *et al.* 1997) and that of cell wall-bound invertase 50 %. These values were used to calculate enzyme activities.

Enzyme assays

After desalting and purification, SUSY activity was measured in 100 µl extract samples added with 100 µl of a reaction mixture containing 100 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 4 mM fructose, and 3 mM UDP glucose (UDPGlc) and that of SPS by adding 100 µl of a reaction mixture containing 100 mM Hepes-NaOH (pH 7.5), 5 mM MgCl2, 1 mM EDTA, 4 mM Fru6P, 20 mM glucose-6P, and 3 mM UDPGlc (Hubbard *et al.* 1989). The mixtures were incubated at 37 °C for 30 min and the reaction stopped by adding 70 µl 30 % KOH. The sucrose produced by these reactions was assayed using the anthrone method (van Handel 1968).

SAI activity was assayed by adding 0.2 cm^3 0.5 mM citrate-phosphate buffer (pH 5.2) and 0.5 cm^3 0.2 M sucrose to 0.3 cm^3 desalted extract and incubating at 37 °C for 1 h. The reaction was stopped by adding 0.2 cm^3 2.5 M Tris base and boiling the mixture for 3 min. Reducing sugars were determined by the Somogyi-Nelson method (Nelson 1944). The assay for NI activity was similar to that of SAI except that the reaction was done in phosphate buffer (pH 7.2).

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard protein. Enzyme activities were calculated on each internode and expressed as micromoles of product formed per gram of total protein per minute. The data presented are the mean values of at least three independent extractions.

Results and Discussion

The concentration of sucrose increased until the 7th month in the Zmex 55-32 variety, but did not in-

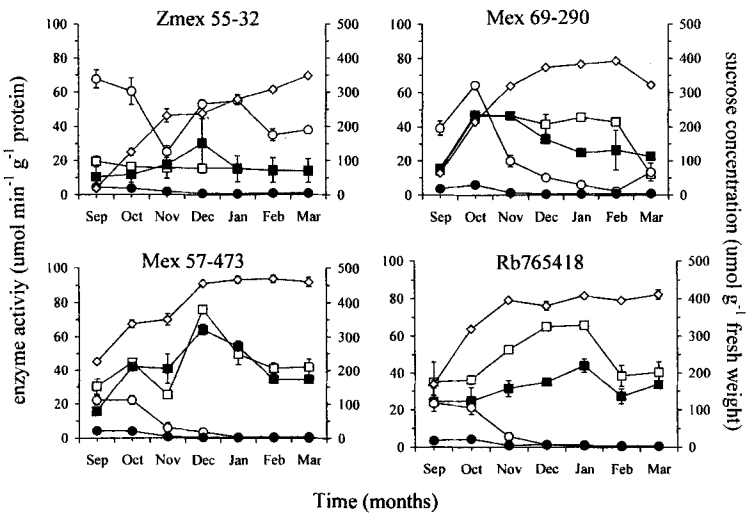

Fig. 1. Changes over time (months) of the sucrose concentration (μmol·g^{-1} fresh weight) (◊) and enzyme activity of (□) sucrose synthase (SUSY), (■) sucrose phosphate synthase (SPS), (○) neutral invertase (NI), and (●) soluble acid invertase (SAI) (μmol·min^{-1}·g^{-1} protein) in stem of different varieties of sugarcane obtained by callus culture.

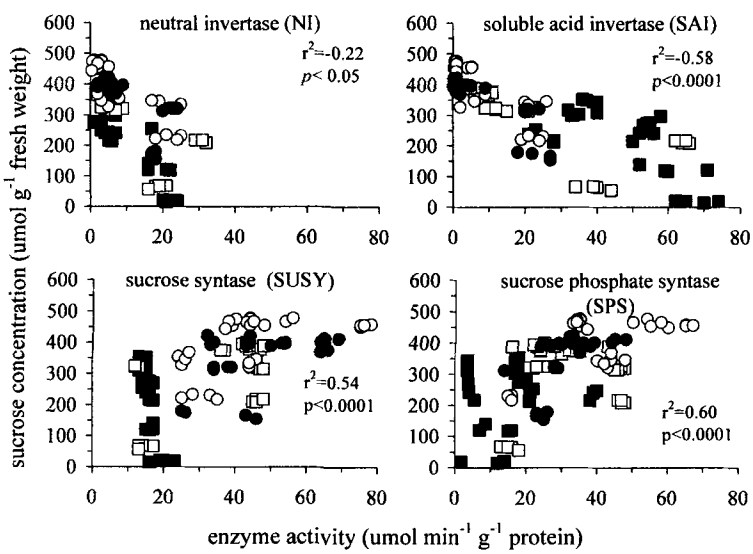

Fig. 2. The relationship between sucrose concentration (μmol·g^{-1} fresh weight) and the activity of sucrose phosphate synthase (SPS), soluble acid invertase (SAI), neutral invertase (NI), and sucrose synthase (SUSY) (μmol·min^{-1}·g^{-1} protein) in the stem of sugarcane varieties ZMex5532 (□), Mex69290 (■), Mex57473 (○), and Rb765418 (●).

crease much more in varieties Mex 57-473 and Mex 69-290 after 4 months, and after 3 months in variety Rb765418 (Fig. 1). The largest concentration of sucrose in stem was found for variety Mex 57-473 and the lowest in Zmex 55-32, as expected. The activity of SPS was largest in variety Mex 57-473 and lowest in Zmex 55-32. The activity of SUSY showed the same pattern as SPS in all varieties. The activity of SAI was low in all varieties compared to the activities of the other enzymes. In varieties Rb 765418 and Mex 57-473, the activity of NI decreased and was low after the 3 month. In the variety Zmex 55-32, the activity of NI decreased until the third month, increased again until the 5th month after which it decreased again. In the variety Mex 69-290, the activity of NI increased until the second month, decreased thereafter and showed a small increase again at the 7th month. Relatively few studies have related changes in sucrose metabolism and its accumulation during ripening. Hatch *et al.* (1963) followed seasonal changes in the activity of several enzymes involved in sucrose metabolism in immature internodes and noted an increase in acid invertase activity with rapid growth and a decrease during sucrose accumulation. The role of invertase has been repeatedly confirmed (Hatch *et al.* 1963, Glasziou and Waldron 1964, Glasziou *et al.* 1966). Seasonal changes in SUSY activity was associated with elongation (Matsui 1985); this finding led to the suggestion that SUSY activity might be associated with the sink strength of the elongating internodes (Lingle and Smith 1991). There was no clear relationship among sucrose accumulation, fructose-2-6-biphosphate concentration, and the activity of Ppi-phospho fructo kinase or ATP-phospho fructo kinase (Lingle and Smith 1991). The activity of SPS and SUSY was greatest when the largest accumulation of sucrose was found.

The accumulation of sucrose in the stem was low when the activity of SAI was high as found by Zhu *et al.* (1997). They reported that in 9-month-old stems SAI played a critical role in limiting sucrose accumulation in sugarcane. No sucrose was accumulated when SAI activity was > 7 μmol·min^{-1}·g^{-1}

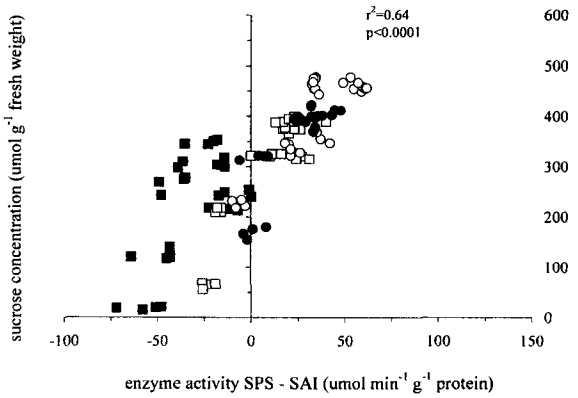

Fig. 3. The relationship between sucrose concentration (μmol·g^{-1} fresh weight) and the activity of sucrose phosphate synthase (SPS) minus the activity of soluble acid invertase activities (SAI) (μmol min^{-1}·g^{-1} protein) in the stem of sugarcane varieties ZMex5532 (□), Mex69290 (■), Mex57473 (○), and Rb765418 (●).

of protein. Zhu *et al.* (1997) also reported that the accumulation of sucrose was low when the activity of NI was high as found in this work.

Sugar accumulation in the sugarcane stem changes over time (Sacher *et al.* 1963, Glasziou and Gayler 1972) and is different between varieties. A model for sucrose accumulation proposed by Moore and Maretzki (1996) established that photoassimilate moves from the phloem to the storage vacuole and the sugars from the vacuole to the cytoplasm, free space and eventually move back into the phloem. The differences between varieties and changes over time can be explained by the direction and flux of sugar movement mediated by the growth rate of the plant and by the changes in activity of invertase which occur in response to variations in temperature and water stress (Hatch and Glasziou 1963), nitrogen status (Sacher *et al.* 1963), diurnal changes in light (Slack 1965), and growth regulators (Su *et al.* 1992).

Plotting the activity of the different enzymes versus the concentration of sucrose gave a significant positive correlation for SPS ($r^2 = 0.60$, $P<0.0001$) and SUSY ($r^2 = 0.54$, $P<0.0001$) and a negative significant correlation for SAI ($r^2 = -0.58$, $P<0.0001$) and NI ($r^2 = -0.22$, $P<0.05$) (Fig. 2). Large activities of SPS and SUSY increased concentrations of sucrose while large activities of SAI and NI had opposite effects. Botha and Black (2000) also found a highly significant positive correlation between SPS activity and sucrose content and Ma *et al.* (2000) stated that SAI had a negative correlation and was important in the accumulation of sucrose. Zhu *et al.* (1997) plotted the activity of SPS minus the activity of SAI, the two most important enzymes for synthesis of sucrose, versus the sucrose concentration. Doing this for the four varieties used in this study gave similar results as those reported. There was a positive and significant correlation and the largest concentrations of sucrose were found when the difference between the two enzymes was most positive (Fig. 3).

Invertase enzymes have been proposed as key regulators for the accumulation of sucrose in sugarcane stem storage parenchyma (Sacher *et al.* 1963, Slack 1965). Later research, however, discounted the importance of invertase (Veith and Komor 1993). Other enzymes such as SUSY (Goldner *et al.* 1991), SPS (Hatch 1964), or the balance of activities of several enzymes (Wendler *et al.* 1990, Veith and Komor 1993, Komor 1994) appeared to be more important. Zhu *et al.* (1997) working with closely related genotypes (progeny) found that differences in the capacity to store sucrose under the same environmental conditions is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase.

It was shown that micropropagation of commercial varieties of sugarcane plants from Mexico had no effect on relative importance of the different enzymes involved in the accumulation of sucrose.

Acknowledgements

The research was funded by the department of Biotechnology and Bioengineering Centro de Investigación y de Estudios Avanzados del I.P.N. (CINVESTAV-IPN, México), and F. G.-M. received grant-aided support from Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico.

References

Botha F.C., Black K.G. 2000. Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. Aus. J. Plant Physiol., 27: 81-85.

Bowen J.E. 1972. Sugar transport in immature internodal tissue of sugarcane. Mechanism and kinetics of accumulation. Plant Physiol., 49: 82–86.

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem., 72: 248-254.

Bull T.A., Glasziou K.T. 1963. The evolutionary significance of sugar accumulation in *Saccharum*. Aust. J. Biol. Sci., 16: 737-742.

Clements H.F., Ghotb A. 1968. The numbering of leaves and internodes for sugarcane nutrition studies. Proc. Int. Soc. Sugar Cane Technol., 13: 569-584.

Dancer J., Hatzfeld W.D., Stitt M. 1990. Cytosolic cycles regulate the turnover of sucrose in heterotrophic cell-suspension cultures of *Chenopodium rubrum*. L. Planta, 182: 223–231.

Glasziou K.T., Gayler K.R. 1972. Storage of sugars in stalks of sugarcane. Bot. Rev., 38: 471-490.

Glasziou K.T., Waldron J.C. 1964. Regulation of acid invertase-levels in sugarcane stalks by auxin and metabolite mediated control systems. Nature, 203: 541-542.

Glasziou K.T., Waldron J.C., Bull T.A. 1966. Control of invertase synthesis in sugarcane: loci of auxin and glucose effects. Plant Pysiol., 41: 282-288.

Goldner W.M., Thom M., Maretzki A. 1991. Sucrose metabolism in sugarcane cell suspension cultures. Plant Sci., 73: 143-147.

Grof C.P.L., Campbell J.A. 2001. Sugarcane sucrose metabolism: scope for molecular manipulation Austr. J. Plant Physiol., 28: 1-12.

Grof C.P.L., Knight D.P., McNeil S.D., Lunn J.E., Campbell J.A. 1998. A modified assay method shows leaf sucrose-phosphate synthase activity is correlated with leaf sucrose content across a range of sugarcane varieties Austr. J. Plant Physiol., 25: 499-502.

Hatch M.D. 1964. Sugar accumulation by sugar-cane storage tissue: the role of sucrose phosphate. Biochem J., 93: 521-526.

Hatch M.D., Glasziou K.T. 1963. Sugar accumulation cycle in sugarcane. II. Relationship of invertase activity to sugar content and growth rate in storage tissue of plants grown in controlled environments. Plant Physiol., 38: 344-348.

Hatch M.D., Sacher J.A., Glasziou K.T. 1963. Sugar accumulation cycle in sugar cane. I Studies on enzymes of the cycle. Plant Physiol., 38: 348–354.

Hubbard N.L., Huber S.C., Parr D.M. 1989. Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon (*Cucumis melo* L.) fruits. Plant Physiol., 91: 1527-1534.

Komor E. 1994. Regulation by futile cycles: the transport of carbon and nitrogen in plants. In: Flux control in biological systems ed. by E.D. Schulze, Academic press, San Diego CA:153-201.

Lingle S.E., Smith R.C. 1991. Sucrose metabolism related to growth and ripening in sugarcane internodes. Crop Sci., 31: 172-177.

Ma H.M., Albert H.H., Paul R., Moore P.H. 2000. Metabolic engineering of invertase activities in different subcellular compartments affects sucrose accumulation in sugarcane cells Austr. J. Plant Physiol., 27: 1021-1030.

Matsui T. 1985. Seasonal changes of the activities of sucrose synthase and sucrose phosphatase synthetase in sugarcane. Tech. Bull. Fac. Agric. Kagawa Univ. 36: 117-125.

Moore P.H. 1995. Temporal and spatial regulation of sucrose accumulation in the sugarcane stem. Aus. J. Plant Physiol., 22: 661–679.

Moore P.H., Maretzki A. 1996. Sugarcane. In: Photoassimilate distribution in plants and crops: Source-sink relationships ed. by E. Zamski, A.A. Schaffer, Marcel Dekker, Inc, New York, Basel, Hong Kong, 643-669.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant, 15: 473-497.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153: 375-380.

Sacher J.A., Hatch M.D., Glasziou K.T. 1963. Sugar accumulation cycle in sugar cane III. Physical and metabolic aspects of cycle in immature storage tissues. Plant Physiol., 38: 348-359.

Slack C.R. 1965. The physiology of sugarcane. VIII. Diurnal fluctuations in the activity of soluble invertase in elongating internodes. Aus. J. Biol. Sci., 18: 781-788.

Su L.Y., Dela Cruz A., Moore P.H., Maretzki A. 1992. The relationship of glyphosate treatment to sugar metabolism in sugarcane: new physiological insights. J. Plant Physiol., 140: 168-173.

van Handel E. 1968. Direct microdetermination of sucrose. Ann. Biochem., 22: 280-283.

Veith R., Komor E. 1993. Regulation of growth, sucrose storage and ion content in sugarcane cells, measured with suspension cells in continuous culture growth under nitrogen, phosphorus or carbon limitation. J. Plant Physiol., 142: 414-424.

Wendler R., Veith R., Dancer J., Stitt M., Komor E. 1990. Sucrose storage in cell suspension cultures of *Saccharum* sp (sugarcane) is regulated by a cycle of synthesis and degradation. Planta, 183: 31–39.

Zhu Y.J., Komor E., Moore P.H. 1997. Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. Plant Physiol., 115: 609-616.

Received March 11, 2002; accepted September 03, 2002