

## EFFECT OF TRANSIENT TEMPERATURE CHANGE ON SUCROSE METABOLISM IN SUGARCANE INTERNODES

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### ABSTRACT

Temperatures can fluctuate rapidly during the sugarcane harvest season, but the effect of short-term temperature changes on sugar metabolism in sugarcane internodes is unknown. This experiment was designed to test the hypothesis that a transient temperature change alters the activity of one or more sucrose cleavage enzymes in sugarcane internodes. In a growth chamber, sugarcane was subjected to a 24-h treatment at 10°C before returning the chamber to a 10-h, 28°C day, 14-h, 18°C night regime. The transient temperature decrease slightly increased sucrose concentration in the most immature internodes sampled for the first two days after initiation of chilling. However, there was no significant change in activities of the sucrose metabolism enzymes soluble acid invertase, neutral invertase, sucrose synthase, sucrose-phosphate synthase, and cell wall acid invertase *in vitro*. The slight increase in sucrose concentration was likely due to low-temperature suppression of sucrose cleavage enzymes *in planta*. Thus, the hypothesis that short-term exposure to cold temperatures alters sucrose metabolism in sugarcane was not supported.

### INTRODUCTION

Sugar concentration in sugarcane is influenced by the balance of sucrose metabolism enzymes in the stem (Moore, 1995; Zhu et al., 1997). The invertases (EC 3.2.1.26; soluble acid, neutral and cell wall-bound acid) contribute to sucrose cleavage, while sucrose-phosphate synthase (SPS; EC 2.4.1.14) synthesizes sucrose. Sucrose synthase (SuSy; EC 2.4.1.13) can either cleave or synthesize sucrose (Geigenberger and Stitt, 1993). The relative activities of these enzymes vary with development (Lingle, 1997; 1999). A number of studies have examined the effect of different growing temperatures on enzyme activity and sugar accumulation in sugarcane. For instance, Alexander and Samuels (1968) showed that immature internodes of sugarcane grown at 13 to 16°C had lower soluble acid invertase content and higher sucrose content than those grown at 27 to 29°C. Similarly, Ebrahim et al. (1998) showed that the activities of soluble acid invertase (SAI), neutral invertase (NI), SuSy and SPS were greater in plants grown at 27°C than at 15°C. However, these studies were done at constant temperature regimes. In sub-tropical and temperate sugarcane growing areas, such as Louisiana, temperatures can fluctuate rapidly and dramatically during the sugarcane harvest season. The effect of these transient temperature changes on sugar metabolism in sugarcane is unknown.

Studies of the effect of environmental changes are best controlled in growth chambers. However, mature sugarcane requires specialized growth chambers. *Dwarf1* is a tissue culture mutant of the cultivar LCP 83-137 characterized by internodes about 2 cm long rather than the 20 cm more typical of normal sugarcane (Burner, 1999). Otherwise, it appears to be identical to LCP 83-137. The short stature of *Dwarf1* makes it suitable for growth chamber experiments.

This study utilized *Dwarf1* to test the hypothesis that a transient temperature change alters sucrose metabolism in sugarcane.

## MATERIALS AND METHODS

### Experimental Design and Plant Culture

The experiment was designed as a split-plot with three replications over time and four subsamples within each replication. For each replicate, 50 one-bud stem pieces of *Dwarf1*, were planted one per 8-L pot containing a 3:1 (v/v) mixture of commercial potting mix and sand. The pots were fertilized with 14 g of Osmocote Plus time-released fertilizer (15-9-12; Scotts-Sierra Horticultural Products Co., Marysville, OH) and placed in a glass house in New Orleans, Louisiana. When plants were approximately 6 months old and had at least 12 above-ground internodes, 40 plants were trimmed to a single, primary stalk. Twenty plants were moved into each of two growth chambers adjusted to a 10 h, 28°C day (0700 to 1700 h) and a 14 h, 18°C night. Humidity was not controlled and fluctuated between 50 % during the light period to 80 % during the dark. Photosynthetic photon flux density at the surface of the upper leaves was about 450  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , achieved with a combination of fluorescent and incandescent bulbs. Temperature and relative humidity in each chamber were monitored and synchronized using Watch Dog data loggers (Spectrum Technologies, Inc., Plainfield, IL).

Each replication of the experiment was started 3 weeks after moving the plants to the growth chambers. At 0900 h on Day 0, four plants (subsamples) were sampled from each chamber. The temperature in one chamber was then adjusted to 10°C, the mechanical limit of the chamber. One day after initiation of the chilling temperatures, four plants were again sampled from each chamber, and temperature in the chilled growth chamber was adjusted back to the 28/18°C temperature regime. Further samples were taken 2, 4, and 8 days after initiation of the treatment.

### Enzyme and Sugar Analysis

Internode 1 of the plants was defined as that subtending the point of attachment of the leaf with the top-most exposed dewlap, which is the youngest fully-expanded leaf. Immediately prior to starting the experiment, that leaf was marked by punching a hole in it with a paper punch. This hole was a marker for identifying the internodes to be sampled. Internodes in positions 2, 4, 6, and 8 at the initiation of the treatment were sampled. The rind was discarded from all internodes except internode 2. Each internode was frozen in liquid N<sub>2</sub>, placed on dry ice for transport to the laboratory, and stored at -80°C. Frozen tissue was crushed in liquid N<sub>2</sub> and stored at -80°C prior to assay.

Enzymes and sugars were extracted in 100 mM HEPES-NaOH (pH 7.5), 0.05 % (w/v) Triton X-100, 5 mM Na-EDTA, 10 mM MgCl<sub>2</sub>, 5 mM DTT by grinding in a mortar with a pinch of sand. Cell wall debris was removed by filtration through cheesecloth (first replication) or centrifuging at 4,200 g for 10 min (subsequent replications). A 0.5 ml aliquot of filtrate or supernatant was mixed with 0.5 ml ethanol and frozen at -80°C for later sugar analysis.

Prior to desalting, 25  $\mu$ l of Protease Inhibitor Cocktail for plant cell extracts (P9599; Sigma-Aldrich, St. Louis, MO) was added to 2.5 ml of extract. This extract was then desalted through a PD-10 column (Amersham Biosciences, Piscataway, NJ) equilibrated with 50 mM Hepes-NaOH (pH 7.5), 0.5 mM Na-EDTA, 5 mM  $MgCl_2$ , and 2.5 mM DTT. Three ml of desalted extract was concentrated to 0.3 ml using an Ultrafree-4 Centrifugal Filter (Millipore Corp., Bedford, MA) with a 10,000 MW cutoff. The concentrate was used for the SPS assay. The remaining, unconcentrated desalted extract was used to assay SAI, NI, and SuSy, and to determine protein.

All enzymes were assayed in triplicate at 37°C. SAI was assayed in 100  $\mu$ l final volume of 50 mM Na-citrate, pH 5.0 and 125 mM sucrose. Reactions were stopped at 0 and 20 min by addition of 100  $\mu$ l 200 mM Tris-HCl, pH 8.0, and boiling 3 min. NI and SuSy were assayed similarly in 50 mM Hepes-NaOH, pH 7.0, 125 mM sucrose, and 2.5 mM  $MgCl_2$ , with (SuSy) or without (NI) 2 mM UDP. For both assays, glucose and fructose standards received the same treatment as reaction tubes. Glucose and fructose concentrations were determined using an enzyme coupled method (Huber and Akazawa, 1986) in a microplate.

SPS activity in concentrated extracts was determined in 100  $\mu$ l final volume 50 mM Hepes-NaOH, pH 7.5, 5 mM  $MgCl_2$ , 1 mM EDTA, 10 mM UDPG, 15 mM glucose-6-phosphate, and 5 mM fructose-6-phosphate. The hexose phosphates were omitted from control tubes. Reactions were stopped by adding 100  $\mu$ l 300 g L<sup>-1</sup> KOH and boiling 3 min. After all reactions were completed, the tubes were heated at 95°C for 15 min to destroy unreacted fructose. Sucrose was determined using the anthrone method (Van Handel, 1968).

In the third replication, cell wall acid invertase (CWAI) was also assayed (Albertson et al., 2001). After centrifuging the extracted tissue slurry, and removing the supernatant for soluble enzymes, the pellet was washed twice by resuspending in 10 mL extraction buffer, placing on ice for 10 min with occasional swirling to mix, followed by 15 min centrifugation at 4,200 g. The final pellet was resuspended in 1.7 ml extraction buffer. A 200  $\mu$ l aliquot of each pellet was added to reaction mixture (25 mM Na-citrate, pH 3.5 and 125 mM sucrose, 800  $\mu$ l final volume) in 1.5 ml microfuge tubes to which three 3 mm glass beads had been added. Tubes were placed on a horizontal shaker at 37°C, at 110 rpm. Reactions were stopped at 0 and 60 min by adding 160  $\mu$ l 4 M imidazole pH 7.6. After centrifuging at 13,000 g 1 min to pellet cell debris, triplicate aliquots of 50  $\mu$ l of the supernatant from each tube were transferred to a microplate, and glucose and fructose determined as described above.

Sucrose, glucose and fructose in the extract were quantified on a Dionex Ion Chromatograph (Dionex Corp., Sunnyvale, CA) using a CarboPac PA1 (Dionex) column with a gradient of 160 to 200 mM NaOH as the eluent. Sugar peaks were detected with a PAD-2 pulsed amperometric detector (Dionex), and quantified by area integration in comparison with external standards. Total sugar concentration was calculated as a sum of sucrose, glucose and fructose concentrations. The ratio of sucrose to total sugar was also calculated.

### Statistical Analysis

The data for each internode were analyzed separately using the PROC MIXED procedure

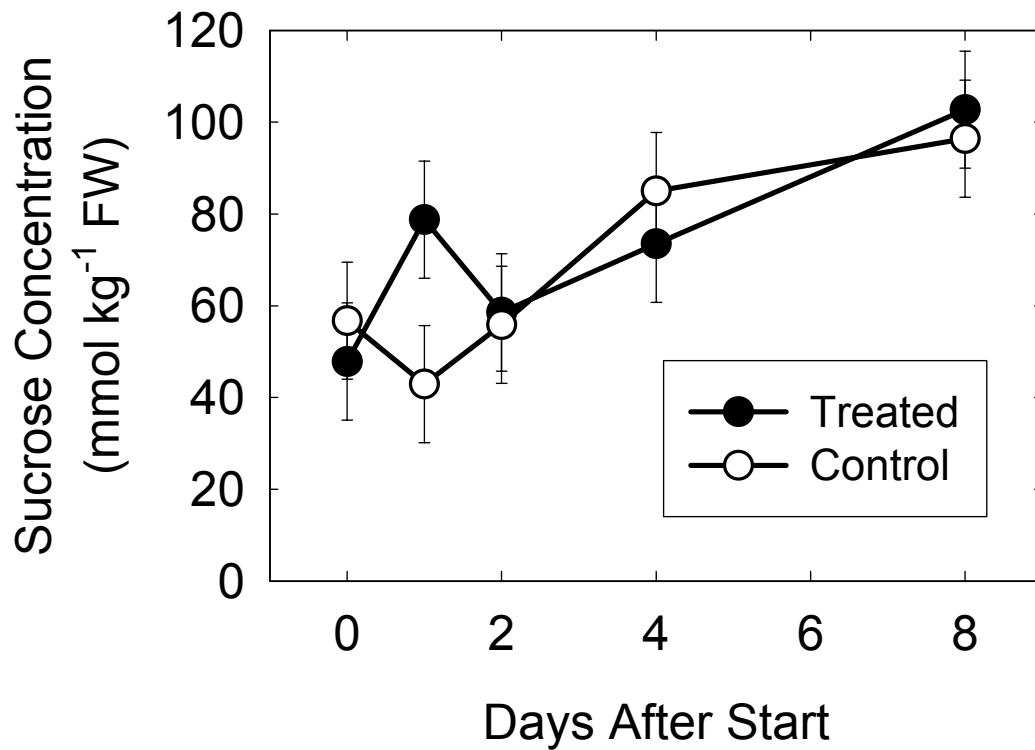
of SAS (SAS Institute, 2001). The different replications of the experiment were treated as blocks, with the temperature treatment as the main plot and time of sampling (days) as subplots. Treatment and days were considered to be fixed effects, while block was considered to be random. Because the variances were proportional with the treatment mean squares, the data for the enzyme, sucrose and total sugar concentration data were transformed using the logarithmic transformation prior to analysis (Steel and Torrie, 1960). The data for the sucrose to total ratio were transformed with the inverse sine transformation.

Cell wall invertase activity was assayed only during the third replication of the experiment. Therefore, it was analyzed as a completely randomized split plot design with the four samples taken each day as replications.

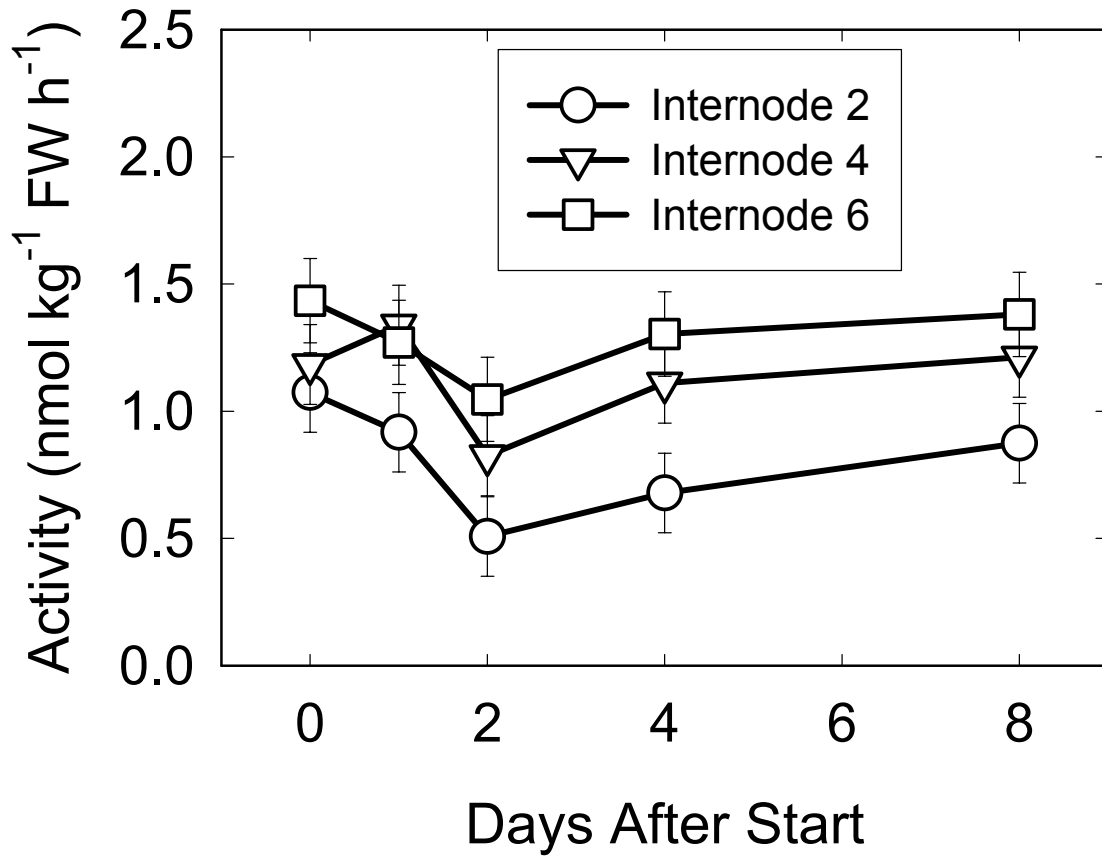
## RESULTS

The main effect of the temperature treatment on sucrose concentration was not significant for any internode (Table 1). However, there was a significant treatment x time interaction for Internode 2. The treatment increased sucrose concentration in Internode 2 sampled one day after the start (Fig. 1). After the temperature was returned to the control setting, the sucrose concentration in Internode 2 of treated plants decreased so that at subsequent samplings it was equivalent to that in control plants. There was an increase in sucrose concentration with time in the other internodes (data not shown). The chilling treatment did not significantly affect the sucrose to total sugar ratio in any internode (Table 1).

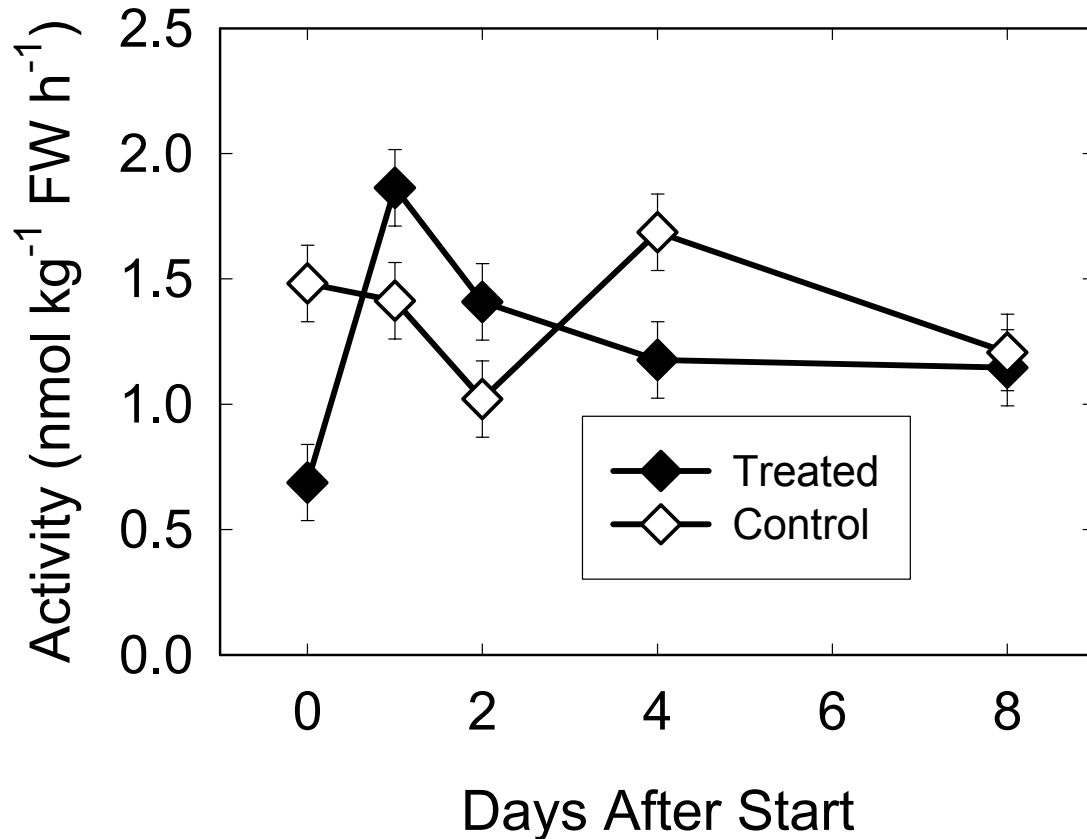
The activities of SAI, NI, SuSy and SPS were greater in internode 2 than the other internodes (data not shown). This has been observed in previous studies (Lingle, 1999). The chilling treatment had no effect on the activity of any of the soluble enzymes. CWAI activity increased with internode age (Fig. 2), but this activity was unaffected by the 24-h chilling treatment, except in Internode 8 (Fig. 3).



**Figure 1.** The effect of 24 h at 10°C on sucrose concentration in Internode 2, counted down from the top-most fully emerged leaf, in *Dwarf1* sugarcane. Vertical bars represent  $\pm$ SE of three replications with four subsamples each.



**Figure 2.** Activity of cell wall acid invertase in *Dwarf1* sugarcane internodes, counted down from the top-most fully emerged leaf.



**Figure 3.** Effect of 24 h at 10°C on cell wall acid invertase activity in Internode 8 of *Dwarf1* sugarcane, counted down from the top-most fully emerged leaf.

### DISCUSSION

This experiment tested the hypothesis that a short-term decrease in temperature alters sugar cleavage and synthesis in sugarcane internodes. Plants were subjected to two 18°C changes in temperature, a decrease at the start of the treatment, and an increase to the control temperature 24 h later. Because temperature has a direct effect on enzyme activity, all enzymes were assayed at 37°C. Thus, any observed differences in activity would be due to changes in enzyme expression, synthesis or modification, and not to a temporary suppression of activity due to the reduced temperature.

Results of this study did not support the hypothesis that a transient change in temperature altered sucrose metabolism in sugarcane. The change in temperature apparently did not affect the activity of soluble sucrose metabolism enzymes in sugarcane internodes. The lack of response of assayed enzyme activity to the temperature treatment suggests that the increase in sucrose concentration observed in Internode 2 (Fig. 1) was due to a transient suppression of sucrose cleavage activity during the 24 h at 10°C.

CWAI has seldom been studied in sugarcane internodes. In this study, CWAI increased

with internode age (Fig. 2), similar to results reported by Botha, et al. (1996). The increase of CWAI activity with internode age is concomitant with the increase in sucrose and sucrose to total sugar ratio (data not shown). This seems counterintuitive. However, Glasziou and Gayler (1972) proposed a cleavage and resynthesis model for sucrose unloading and storage in sugarcane. In this model, sucrose is unloaded into the apoplast of the storage parenchyma, where it is cleaved into glucose and fructose. The hexoses are then transported into the cell, where sucrose is resynthesized. Lingle (1989) demonstrated that sucrose could be transported intact in sugarcane, but did not show that cleavage and resynthesis played no role in sucrose storage. It remains possible that cell wall acid invertase contributes to sucrose storage in sugarcane. This is currently being pursued.

### ACKNOWLEDGEMENTS

The author thanks Ms. April Allen for performing the enzyme assays, and Ms. Debbie Boykin for assistance with the statistics.

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**Table 1.** Analysis of variance of fixed effects for log transformation of sucrose and total sugar concentration and activity of five sucrose metabolism enzymes, and arcsine of square root transformation of sucrose/total sugar ratio. Plants were treated by chilling to 10°C for 24 h, and sampled at 0, 1, 2, 4 and 8 days after treatment. Internodes were counted down from the top-most fully emerged leaf.

Source	df	Sucrose	Total sugar	Ratio	Soluble acid invertase	Neutral invertase	Sucrose synthase	Sucrose-P synthase	Cell wall invertase
----- P > F -----									
<u>Internode 2</u>									
Treatment (T)	1	0.956	0.838	0.800	0.598	0.239	0.118	0.180	0.179
Days (D)	4	<0.001	0.004	<0.001	<0.001	0.008	0.005	0.003	0.901
T x D	4	0.007	0.054	0.227	0.030	0.678	0.739	0.200	0.317
<u>Internode 4</u>									
Treatment (T)	1	0.274	0.194	0.727	0.410	0.888	0.985	0.948	0.260
Days (D)	4	<0.001	<0.001	<0.001	0.539	0.603	0.066	0.114	0.650
T x D	4	0.428	0.508	0.774	0.629	0.274	0.886	0.192	0.259
<u>Internode 6</u>									
Treatment (T)	1	0.388	0.375	0.604	0.801	0.290	0.872	0.474	0.618
Days (D)	4	<0.001	<0.001	<0.001	0.031	0.182	0.027	0.075	0.528
T x D	4	0.544	0.550	0.799	0.370	0.564	0.438	0.420	0.872
<u>Internode 8</u>									
Treatment (T)	1	0.735	0.705	0.547	0.154	0.290	0.049	0.676	0.004
Days (D)	4	0.019	0.025	<0.001	0.236	0.310	0.132	0.643	0.167
T x D	4	0.349	0.302	0.305	0.425	0.491	0.997	0.261	<0.001