Relationship of Acid Invertase Activities to Sugar Content in Sugarcane Internodes during Ripening and after Harvest

W. Lontom[1], M. Kosittrakun[1,]* and S.E. Lingle[2]

[1]*Applied Taxonomic Research Center, Department of Biology, Faculty of Science Khon Kaen University, Khon Kaen 40002, Thailand*
[2]*USDA-ARS, Southern Regional Research Center, 1100 Robert E. Lee Blvd. New Orleans, LA 70124, USA*

**Corresponding author. Email: mankos@kku.ac.th*

Abstract

It has been hypothesized that soluble acid invertase (SAI) and insoluble (cell wall) acid invertase (CWI) influence sucrose accumulation in sugarcane during ripening, and also sucrose breakdown after harvest. The activities of SAI and CWI were determined in selected immature and mature internodes during ripening and during postharvest storage at 18^{o}C in two Louisiana sugarcane cultivars, HoCP 96-540 and L 97-128 grown in Louisiana, USA in 2004. SAI and sugars were extracted from each internode in extraction buffer (100 mM Na-Hepes (pH 7.5), 5 mM Na-EDTA, 10 mM $MgCl_2$, 0.05% Triton X-100 and 5 mM DTT). CWI was solubilized from cell wall residue in the same buffer with the addition of 1 M NaCl. Sucrose, glucose and fructose were quantified by high pressure ion chromatography with pulsed amperometric detection. Total sugar content was calculated as the sum of the molar concentrations of sucrose, glucose and fructose. During ripening, the activity of SAI was highest in the youngest internode. It decreased with internode age. In contrast, the activity of CWI increased as the internode became older. Sucrose content during ripening was negatively correlated with SAI activity in both cultivars (r = -0.449** and -0.503** in HoCP 96-540 and L 97-128, respectively) as was the sucrose-to-total sugar ratio (r = -0.548** and -0.581**). After harvest, the activity of SAI in the immature internode increased with time in storage whereas that of CWI decreased in both immature and mature internodes. There was also a negative correlation between SAI activity and sucrose content (r = -0.426** and -0.493**) and the sucrose-to-total sugar ratio (r = -0.507** and -0.439**) during storage. The data from this study support the hypothesis that acid invertases play key roles in determining sucrose concentration during ripening and after harvest in sugarcane internodes.

Keywords: soluble acid invertase, cell wall acid invertase, sucrose metabolism, sugarcane, ripening, postharvest

Introduction

Sucrose is an important component of yield in sugarcane (*Saccharum* spp.) (Lingle and Smith, 1991). The quality of sugarcane juice at harvest is determined by the concentration of sucrose, which should be high, and the concentration of nonsucose components such as other sugars, which should be low (Lingle, 1997). Sugar begins accumulating in sugarcane internodes when they start elongating and continues until after elongation ceases (Lingle and Smith, 1991). During ripening, sucrose concentrations along the entire stalk increase, and the proportion of the stalk containing significant glucose and fructose concentrations decreases (Fernandes and Benda, 1985). This pattern implies that sucrose metabolism in the stalk also changes during development.

Primary sucrose metabolism is governed by several enzymes (Quick and Schaffer, 1996) such as invertase (E.C.3.2.1.26), sucrose synthase (E.C.2.4.1.13) and sucrose-phosphate synthase (E.C. 2.4.1.14). Invertases (β-fructofuranosidase) have been suggested to be key regulators for sucrose accumulation in sugarcane stem parenchyma (Hatch and Glasziou, 1963; Sacher et al., 1963; Gayler and Glasziou, 1972; Zhu et al., 1997). Two groups of acid invertase, soluble and cell wall bound, are present in sugarcane tissues (Gayler and Glasziou, 1972; Vattuone et al., 1981). These isozymes hydrolyze sucrose into glucose and fructose, but at a much slower rate. They can also remove terminal β-fructosyl residues from short-chain oligosaccharides such as raffinose and kestoses (Avigad and Dey, 1997).

Soluble acid invertase (SAI) is localized in the vacuole (Sturm, 1999; Grof and Campbell, 2001) whereas cell wall invertase (CWI) is localized in the apoplast, ionically linked to the cell wall (Albertson et al., 2001). The activity of SAI is usually high in tissues that are rapidly growing such as cell and tissue cultures, root apices and immature stem internodes (Hatch and Glasziou, 1963). It is also thought to mediate remobilization of sucrose from storage for maintaining cellular processes during periods of the stress such as delayed harvest (Grof and Campbell, 2001). It has been reported that the inversion of sucrose occurs in harvested sugarcane internodes (Salunkhe and Desai, 1988; Mao et al., 2006). In sugarbeet roots, sucrose is lost during postharvest storage due to the continuing metabolic activity of living cells and the presence of the endogenous enzymes capable of degrading sucrose (Wyse, 1974).

We conducted this study to determine the relationship of SAI and CWI activities to sucrose accumulation during ripening and sucrose degradation after harvest in two Louisiana sugarcane cultivars, HoCP 96-540 and L 97-128. The information from this study may aid in the selection or genetic manipulation of sugarcane cultivars for more efficient production.

Materials and Methods

Plant Materials

Whole-stalks of two commercial sugarcane cultivars, HoCP 96-540 and L 97-128, were planted in October 2003 at the USDA-ARS Ardoyne Research Farm near Schriever, LA (N 29 38' 15" W 90 50' 21"). The soil was Cancienne silt loam and Cancienne silty clay loam (fine-silty, mixed, superactive, nonacid, hyperthermic Fluvaquentic Epiaquepts). Plots were fertilized with 112 kg ha^{-1} N, 15 kg ha^{-1} P, and 55 kg ha^{-1} K in the spring of 2004. 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 (Lingle, 2004). Internodes 2, 5, 8 and 11 of each cultivar were marked on 22 October 2004 by clipping two small holes in the attached leaf with a hole-punch. During the ripening period, 3 replications of each internode were sampled on 22 October, 5 November and 19 November 2004. On 29 November 2004, 20 stalks of each cultivar were harvested and stored at 18^{o}C. Three replications of internodes 2 and 11 were sampled on days 0, 2, 4, 7, 11 and 15 after harvest. The node and rind were discarded from all internodes except internode 2. The cylinder of storage tissue was divided into quarter lengthwise wedges. Each wedge was chopped into small pieces, frozen in liquid nitrogen, placed on dry ice for transport to the laboratory and stored at -80^{o}C.

Enzyme Extraction and Assay

Approximately 2-3 g of frozen tissue was ground to powder in liquid nitrogen. Soluble enzyme and sugars were extracted in 8 ml of 100 mM HEPES-NaOH (pH 7.5), 0.05% (w/v) Triton X-100, 5 mM Na-EDTA, 10 mM $MgCl_2$ and 5 mM DTT by grinding in a mortar with a pinch of acid-washed sand. Cell wall debris was removed by centrifuging at 4200 *g* for 15 min at 4^{o}C. After recording the volume, a 0.5-mL aliquot of supernatant was mixed with 0.5 mL ethanol and frozen at -80^{o}C for later sugar analysis. SAI was concentrated by acetone precipitation. The pellet was re-solubilized in 250 μL of 50 mM HEPES-NaOH (pH 7.5), 0.5 mM Na-EDTA, 5 mM $MgCl_2$ and 2.5 mM DTT.

Following the extraction of soluble enzyme, cell wall invertase was solublized by incubating the cell wall residue in 100 mM HEPES-NaOH (pH 7.5), 0.05% (w/v) Triton X-100, 5 mM Na-EDTA, 10 mM $MgCl_2$, 5 mM DTT and 1 M NaCl overnight at 4°C.

Both enzymes were assayed in triplicate for 20 min at 37^{o}C. SAI was assayed by adding 25 µL extract in a final volume of 100 µL of 50 mM Na-citrate, pH 5.0 and 125 mM sucrose. Reactions were stopped at 0 and 20 min by adding 100 µL of 200 mM Tris-HCl, pH 8.0 and boiling for 3 min. CWI was assayed similarly in 50 mM Na-citrate, pH 3.5 and 125 mM sucrose. Glucose and fructose concentrations in the reaction mixture were determined using an enzyme coupled method (Huber and Akazawa, 1986) in a microplate.

Sugar Analysis

Sucrose, glucose and fructose levels of the tissue 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 amperometic detector (Dionex), and quantified by area integration in comparison with external standards. Total sugar content was calculated as a sum of sucrose, glucose and fructose concentrations. The ratio of sucrose-to-total sugars was also calculated.

Protein Determination

Protein concentration of the enzyme extracts were determined by a Quick Start™ Bradford protein assay kit (Bio-Rad Laboratories, Richmond, CA) in a microplate. BSA was used as the standard protein.

Statistical Analysis

Data from each cultivar were analyzed separately. The relationship of enzyme activities with sugar content was analyzed in terms of correlation using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL).

Results and Discussion

Ripening

During the ripening period, SAI activity of both cultivars decreased with internode age (Figures 1A, 1B). It reached the highest level in internode 2 collected on 5 November. The lowest activity was found in both internodes 8 and 11. The change in SAI activity in sugarcane internodes sampled at different days had a similar pattern, with higher activity in younger internode. Zhu et al. (1997) also reported that SAI activity varied by internode age, with highest activity in the young internode of cultivar Molokai 5829 and it declined rapidly, reaching basal levels by internode 3 or 5, as the internodes matured and accumulated sucrose. In contrast, the activity of CWI increased with internode age but the pattern was inconsistent between cultivars (Figures 1C, 1D). In HoCP 96-540 sampled on 22 October, CWI increased with internode age until internode 8, and then reached a plateau (Figure 1C). On the other sampling dates, CWI activity was slightly higher in internodes 5, 8 and 11 compared to internode 2 of HoCP 96-540. In L 97-128, there was a large increase in CWI between internodes 2 and 5 sampled on 5 November, but the other internodes had lower activity (Figure 1D). At the other sampling dates, there was a small increase in CWI with internode age. An increase in CWI with internode age has been reported by Vorster and Botha (1999) and Lingle (2004).

In both sugarcane cultivars, sucrose concentration during the ripening period was low in the immature internode, increasing with internode age (Figures 2A, 2B). In both cultivars, the sucrose concentration of internode 2 increased with each sampling date, an indication of ripening. The greatest sucrose content in internode 11 was slightly higher in HoCP 00-950, 198.2 mol kg^{-1} FW, than in L 97-128, 187.6 mol kg^{-1} FW. Ripening in sugarcane is influenced by conditions unfavorable for growth such as cool temperatures, restricted soil moisture and low available soil nitrogen (Humbert, 1968).

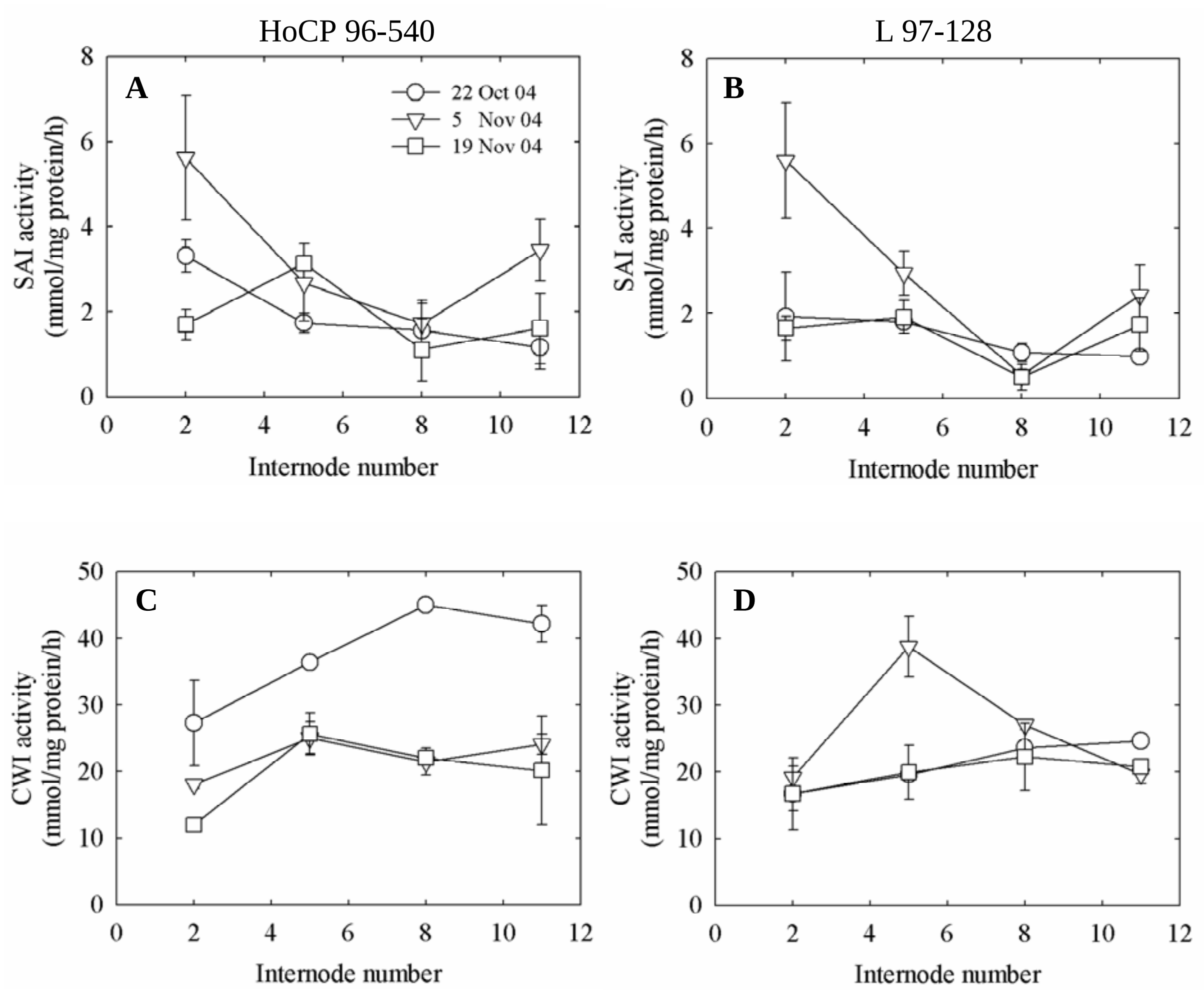

Figure 1 Changes in the activity of soluble acid invertase (A and B) and cell wall acid invertase (C and D) during the ripening period of two sugarcane caltivars. On 22 October 2004, internodes 2, 5, 8 and 11 from the top were tagged. These same internodes were again sampled on 5 November 2004 and 19 November 2004. Bars indicate ± SE.

Ripening can also be demonstrated by the ratio of sucrose-to-total sugar (Figures 2C, 2D). This ratio was lowest in internode 2, and increased to near 1.0 in the older internodes. In both cultivars, the sucrose-to-total sugar ratio in the internode 2 increased with sampling date. Since the upper, immature internodes are the source of most of the impurities in whole-stalk juice, this increase in the proportion of sugars that is sucrose as the season advances improves juice quality in the stalk as a whole. Lingle and Irvine (1994) also reported that sucrose percent of total sugars was strongly affected by sampling date.

Postharvest Storage

In the sugarcane stalks stored at 18^{o}C, the activity of SAI in internode 2 did not change during the first four days, but increased steadily after 7 days of storage in HoCP 96-540 and 4 days in L 97-128 (Figures 3A, 3B). The highest activity of 21.75 mmol mg^{-1} protein h^{-1} was found in internode 2 of cultivar L 97-128 stored for 15 days (Figure 3B). This is in good agreement with the report by Mao et al. (2006), who found that in the sugarcane stalks stored at 20^{o}C, the activity of SAI increased rapidly, reaching the highest activity after 15 days, as the stalks accumulated the highest level of

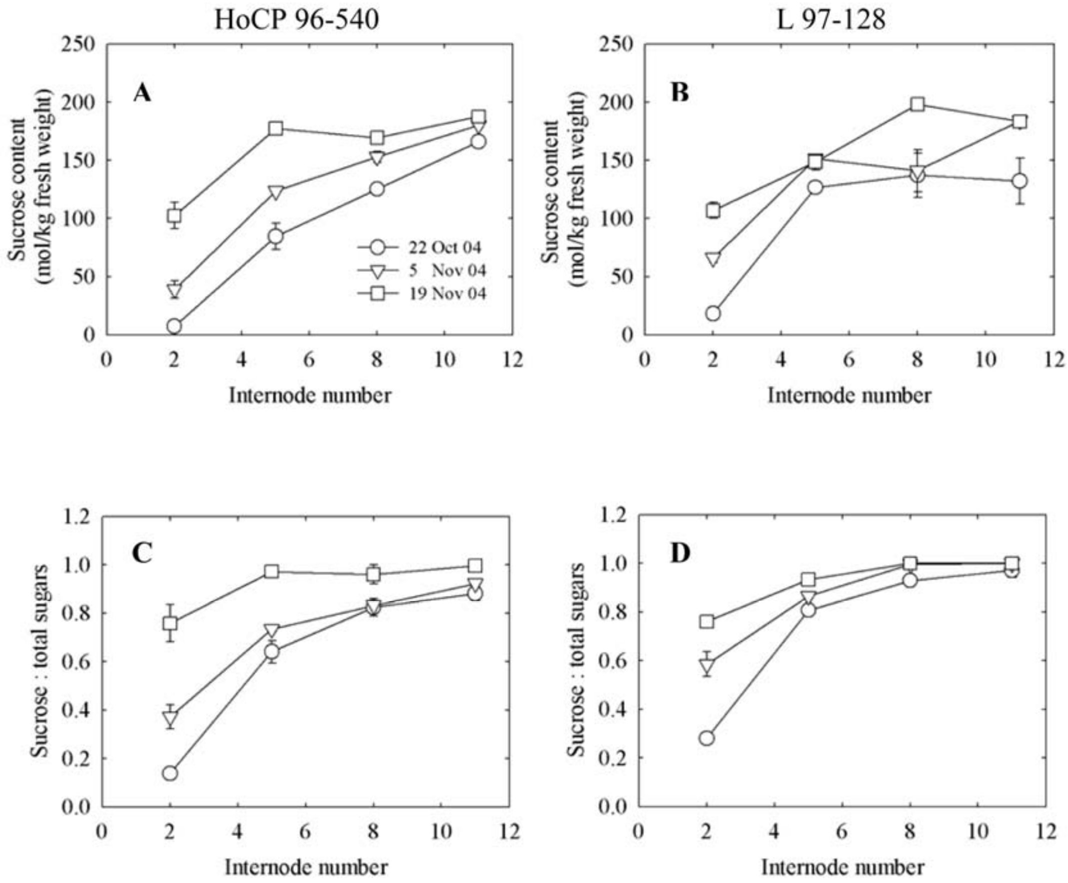

Figure 2 Changes in sucrose content (A and B) and the sucrose-to-total sugar ratio (C and D) during the ripening period of two sugarcane caltivars. On 22 October 2004, internodes 2, 5, 8 and 11 from the top were tagged. These same internodes were again sampled on 5 November and 19 November 2004. Bars indicate ± SE.

reducing sugars. The increase in SAI activity in immature sugarcane internodes after harvest may indicate its necessity for the postharvest generation of respiratory substrates. There was little change in SAI activity of internode 11, which was mature and stored a high concentration of sucrose (Figures 3A, 3B). In some mature sink tissues, such as sugarbeet roots, acid invertase was barely detectable at harvest but increased almost 30-fold during storage at 21°C (Wyse, 1974), but this does not appear to be true for mature sugarcane internodes.

The pattern of a change in CWI activity was different from that in SAI activity. The former was greater in internode 11 than in internode 2 (Figures 3C, 3D). There was an increase in CWI of both internodes by 4 days after harvest. In HoCP 96-540, CWI activity declined to initial levels in both internode (Figure 3C), while in L 97-128, CWI activity of internode 11 remained elevated until 11 days after harvest, and CWI activity in internode 2 declined slowly after 4 days (Figures 3C, 3D).

We hypothesized that after sugarcane stalks were harvested, sucrose content would decrease due to inversion by invertases. However, we discovered that sucrose content changed little during 15 days of storage at 18°C. The sucrose content of internode 2 of HoCP 96-540 was unchanged (Figure 4A), while the sucrose content of internode 2 of L 97-128 increased slowly after 4 days of storage (Figure 4B). Sucrose content of internode 11 increased slightly in the first 2 days after harvest (Figure 4A), while that of L 97-128 decreased slightly after 11 days of storage (Figure 4B). The sucrose-to-total sugar ratio in mature internodes showed a similar

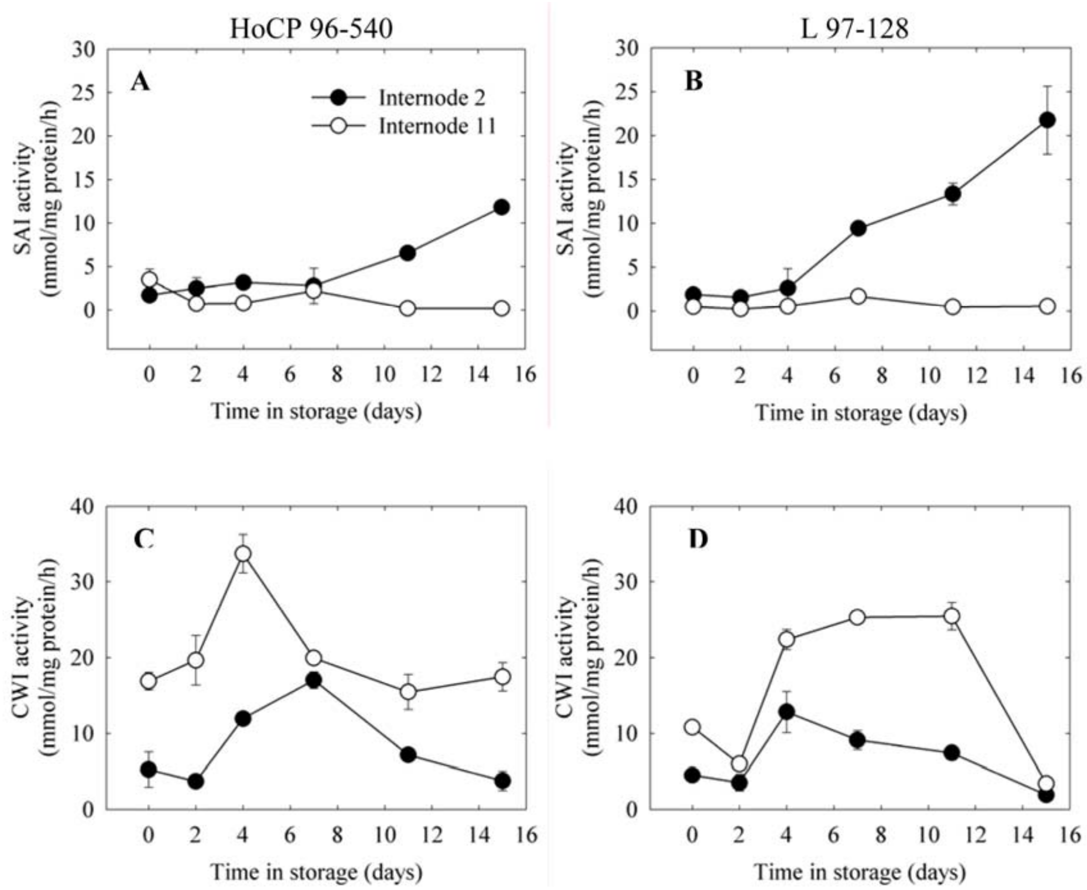

Figure 3 Postharvest changes in the activity of soluble acid invertase (A and B) and cell wall acid invertase (C and D) of internodes 2 and 11 of two sugarcane cultivars during storage at 18°C. Bars indicate ± SE.

pattern: virtually unchanged in HoCP 96-540 (Figure 4C), and a small decrease in L 97-128 (Figure 4D). There were slight increases and decreases in the sucrose content of internode 2 of HoCP 96-540 during storage (Figure 4A), and the sucrose:total sugar ratio similarly decreased and increased (Figure 4C), while in L 97-128, the sucrose content approximately doubled between 2 and 15 days after storage (Figure 4B) and the sucrose:total sugar ratio increased after an initial decrease after 2 days of storage.

Relationship of Acid invertase Activities to Sugar Content

The correlations of acid invertase activities to sucrose content and the sucrose-to-total sugar ratio were analyzed using Pearson correlation in SPSS 11.5 for Windows. The results showed that sucrose content during ripening was negatively correlated with SAI activity in both cultivars (r = -0.449** and -0.503** in HoCP 96-540 and L 97-128, respectively) as was the sucrose-to-total sugar ratio (r = -0.548** and -0.581**) (Table 1). Ma et al. (2000) revealed that SAI had a negative correlation and was important in the accumulation of sucrose. Zhu et al. (1997) found that sucrose accumulation in sugarcane stalks was low when SAI activity was high. The enzyme played a critical role in limiting sucrose accumulation in the 9-month-old sugarcane. Its activity had a negative hyperbolic relationship with sucrose concentration. No sucrose was accumulated when SAI activity was greater than 7 μmol min^{-1} g^{-1} protein. Although positive correlations of CWI activity with sucrose content

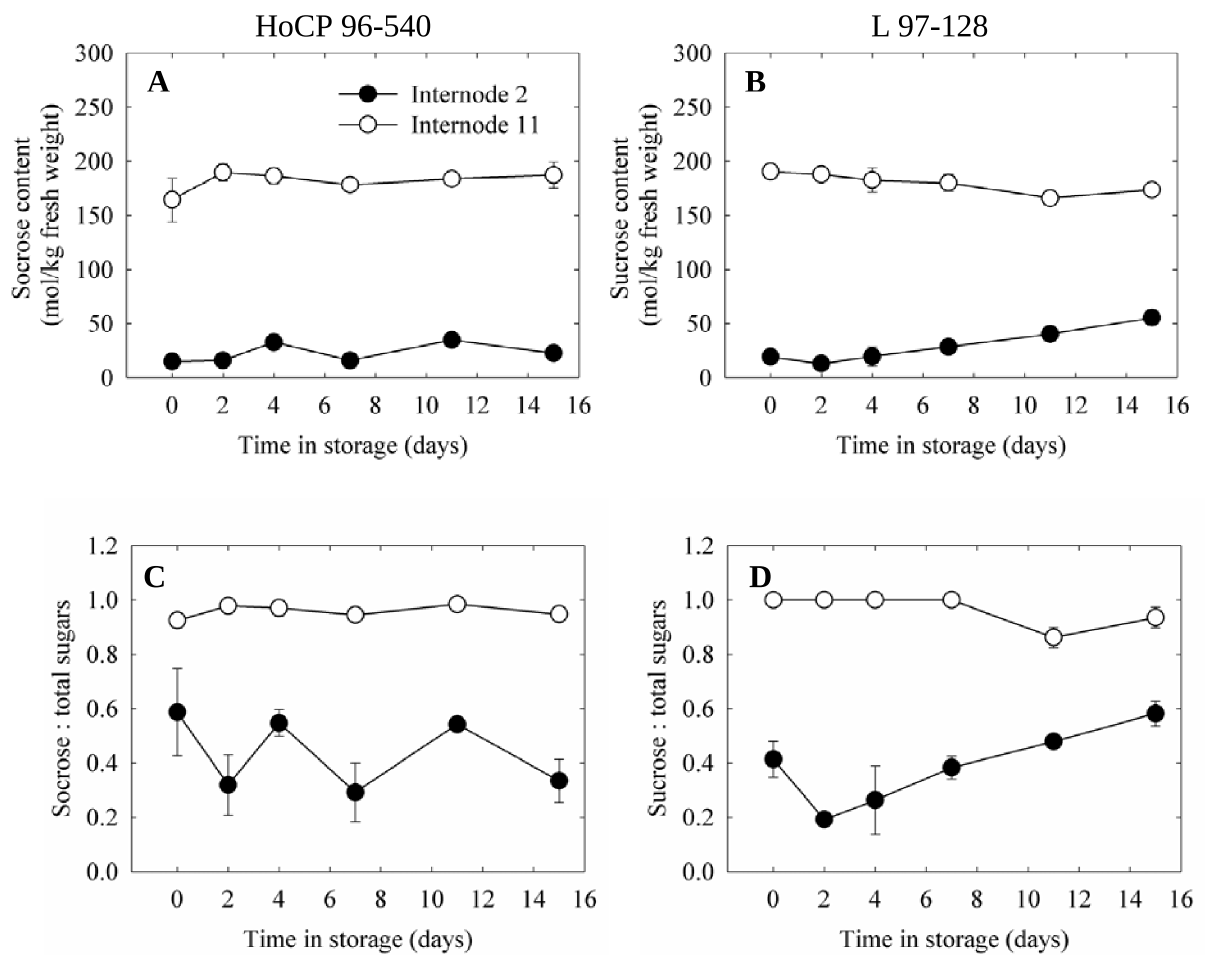

Figure 4 Postharvest changes in sucrose content (A and B) and the sucrose-to-total sugar ratio (C and D) of internodes 2 and 11 of two sugarcane cultivars during storage at 18 ^{o}C. Bars indicate ± SE.

and the sucrose-to-total sugar ratio were found in cultivar L 97-128, the correlation coefficient was relatively low (Table 1).

After harvest, there was also a negative correlation between SAI activity and sucrose content (r = -0.426** and -0.493**) and the sucrose-to-total sugar ratio (r = -0.507** and -0.439**) during storage. However, despite the increase in SAI activity in internode 2 after harvest (Figures 3A, 3B), sucrose concentration was unchanged in HoCP 96-540, and increased slightly in L 97-128 (Figures 4A, 4B), and the sucrose-total sugar ratio increased in Internode 2 of L 97-128 (Figure 4D). This suggests that the mechanisms of sucrose synthesis were still active after harvest, although we did not measure sucrose synthetic enzymes. Mao et al. (2006) indicated that storage of sugarcane internodes seemed to coincide with a redirection of carbon from sucrose to reducing sugars and organic acids and respiration, but the lack of significant loss of sucrose on storage in this study seems to contradict that hypothesis. We calculated sucrose content on a fresh weight basis. Fresh weight would have decreased during storage, although internodes were not weighed when sampled. It is possible that dehydration of the tissue caused some of the apparent increase in sucrose contents, and that sucrose content per internode might have decreased, but that is unknown.

Table 1 Simple correlation of enzyme activities with sucrose content and the sucrose-to-total sugar ratio in the internodes of two sugarcane cultivars during ripening and after harvest[1/].

Enzyme activity	Ripening period		Postharvest period	
	Sucrose content	Sucrose/Total sugars	Sucrose content	Sucrose/Total sugars
HoCP 96-540 *n=36*	---------- *r* ----------			
SAI	-0.449**	-0.548**	-0.426**	-0.507**
CWI	-0.152	-0.086	0.622**	0.509**
L 97-128 *n=36*				
SAI	-0.503**	-0.581**	-0.493**	-0.439**
CWI	0.399*	0.370*	0.502**	0.422**

[1/] *, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Conclusions

During ripening, SAI activity decreased with internode age whereas CWI activity and sucrose content increased. The internodes sampled later in the growing season accumulated more sucrose than those tagged earlier. Based on the correlation data, it can be concluded that SAI activity was associated with sucrose accumulation in sugarcane internodes during the ripening period.

It is evident that harvested sugarcane stalks possessed high SAI activity in young internodes and high CWI activity in mature internodes. Both SAI and CWI activities were correlated with sucrose degradation during storage, but on a fresh weight basis, sucrose loss was minimal, even after 15 days storage at 18°C.

The data from this study support the hypothesis that acid invertases play key roles in determining sucrose concentration during ripening and after harvest in sugarcane internodes.

Moreover, the correlation of enzyme activities with sucrose content might be useful in the selection or genetic manipulation of sugarcane cultivars for more efficient production.

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